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Rectal Absorption of Penicillin. Emanuel E. Mandel, M.D., Chicago, Ill., and James D. Thayer, Ph.D., Staten Island, N. Y. ---------- 135

Parenteral Nutrition. III. Studies on the Tolerance of Dogs to Intravenous Administration of Fat Emulsions. Harvey S. Collins, M.D., Lisbeth M. Kraft, D.V.M., Thomas D. Kinney, M.D., Charles S. Davidson, M.D., John Young, M.D., and Fredrick J. Stare, M.D., Boston, Mass. ---------- 143

Parenteral Nutrition. IV. Improved Techniques for the Preparation of Fat Emulsions for Intravenous Nutrition. Robert P. Geyer, Ph.D., George V. Mann, M.D., and Fredrick J. Stare, M.D., Boston, Mass. ---------- 153

Parenteral Nutrition. V. Studies on Soybean Phosphatides as Emulsifiers for Intravenous Fat Emulsions. Robert P. Geyer, Ph.D., George V. Mann, M.D., John Young, M.D., Thomas D. Kinney, M.D., and Fredrick J. Stare, M.D., Boston, Mass. ---------- 163

Parenteral Nutrition. VI. Fat Emulsions for Intravenous Nutrition: The Turbidimetric Determination of Infused Fat in Blood After Intravenous Administration of Fat Emulsions. Robert P. Geyer, Ph.D., George V. Mann, M.D., and Fredrick J. Stare, M.D., Boston, Mass. ---------- 175

Studies on the Conglutination Test in Erythroblastosis Fetalis. Alexander S. Wiener, M.D., and Eve B. Gordon, Brooklyn, N. Y. ---------- 181

A Study of Cholinesterase Activity of the Blood of Patients With Pernicious Anemia. Leo M. Meyer, M.D., Arthur Sawitsky, M.D., Norton D. Ritz, M.D., and Howard M. Fitch, Ph.D., Brooklyn, N. Y. ---------- 189

A Study of Cholinesterase Activity in the Blood of Normal Subjects. Arthur Sawitsky, M.D., Howard M. Fitch, Ph.D., and Leo M. Meyer, M.D., New York, N. Y. ---------- 203

A Comparison of Methods Used for the Hydrolysis of Conjugated Urinary Estrogens. John T. Van Bruggen, Ph.D., St. Louis, Mo. ---------- 207

Studies on the Chemotherapy of Filariasis. VI. Some Pharmacodynamic Properties of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride, Hetrazan. B. K. Harned, Raymond W. Cunningham, Sybella Halliday, R. E. Vessey, N. N. Yuda, Mary C. Clark, Carolyn H. Hine, Rachel Cosgrove, and Y. SubbaRow, Pearl River, N. Y. ---------- 216

(Contents continued on inside front cover)

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Laboratory Methods

Screening Method for Blood Glucose. Hugh L. C. Wilkerson, M.D., and Erich Heftmann, Ph.D., Boston, Mass. ---------- 236

An Increase of Complement Units by the Use of Egg Albumin. S. W. Bohls, M.D., and Phyllis Shaw, B.S., M.T., (ASCP), San Antonio, Texas ---------- 239

A Colorimetric Method for the Determination of Microquantities of Ethanol in Blood and Other Biologic Fluids. Richard J. Henry, M.D., Carol F. Kirkwood, A.B., Sam Berkman, Ph.D., Riley D. Housewright, Ph.D., and Jane Henry, Ph.D., Frederick, Md. With the Technical Assistance of Renate Hirsch ---------- 241

The Microcolorimetric Determination of Sodium in Human Biologic Fluids. Anthony A. Albanese, Ph.D., and Marilyn Lein, New York, N. Y.---------- 246

A New Colorimetric Method for the Determination of Pregnandiol. Joseph W. Goldzieher, M.D., New York, N. Y. ---------- 251

The Effect of Chlorine on Complement Fixation. Anne C. Kimball, Ph.D., and Paul W. Kabler, Ph.D., M.D., Minneapolis, Minn. ---------- 254

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RECTAL ABSORPTION OF PENICILLIN

EMANUEL E. MANDEL, M.D.,* CHICAGO, ILL., AND
JAMES D. THAYER, PH.D.,† STATEN ISLAND, N. Y.

SINCE the early days of the penicillin era destruction by fecal enzymes has been regarded as one of the main obstacles to effective low intestinal absorption of penicillin.[1-10] Results of more recent investigations on this subject are contradictory. Absorption from ligated loops of the colon of rats was determined[11] to be nearly the same as that from the duodenum. Other authors[6] reported the penicillin uptake from isolated segments of the large intestine of cats to be inferior to duodenal absorption. In a man with a fistula of the colon, penicillin activity in blood and urine was negligible (2.3 per cent urinary recovery) after introduction through the fistula of 0.3 million units in solution.[6] In apparent contrast to these observations, Loewe and associates[12] obtained therapeutic serum concentrations with the rectal use of penicillin suppositories (up to 0.77 unit per milliliter one hour after insertion of one million units). The statement by Davison[13] that "rectal suppositories will maintain a therapeutic level for twenty-four hours" is probably based on those findings. Stimulated by this same report, Barach and co-workers[14] introduced penicillin in aqueous solution rectally only to find no demonstrable blood level. Nevertheless, a paper from the Mayo Clinic[15] which appeared while the present study was in progress listed results comparable to Loewe's, as produced with penicillin suppositories.

In view of these discrepancies and because of the lack of conclusive information on quantitative penicillin absorption from the rectum, the present work was undertaken.

EXPERIMENTS AND METHODS

All experiments were performed on adult male human subjects between the ages of 18 and 50 and on two children, as indicated below, all of whom had no apparent organic intestinal disorder. Penicillin was administered rectally in cocoa butter suppositories, cocoa butter capsules, gelatine capsules, and by microclyster and insufflation, and the resulting antibiotic activity of blood and urine was determined. For reasons of comparison parenteral and oral administration were also studied.

The suppositories (Table I) were made by mixing dry penicillin with cocoa butter at 50 to 60° C. to which was added a small amount of peanut oil or kaolin in the first few trials and 2 per cent beeswax in the later cases. The streptomycin suppositories used in four

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*Surgeon (R), U. S. Public Health Service; Chief of Medical Service, U. S. Marine Hospital.

†Bacteriologist, U. S. Public Health Service, Venereal Disease Research Laboratory, U. S. Marine Hospital.

trials (Table I, Cases 12 to 15) were prepared in the same manner; two such suppositories, each containing 0.25 Gm., were inserted two hours before a penicillin suppository. A cleansing saline enema was given in this and in the subsequent group of experiments one to three hours before the test to those subjects in whom satisfactory elimination had not occurred earlier the same morning. No such enemas were used in any of the following experiments.

A microclyster (Table II) was prepared by dissolving penicillin in physiologic saline (100,000 units per 5 ml.). Injection into the rectum was performed through a rubber catheter, the patient remaining on the side in order to prevent leakage.

Cocoa butter capsules were made by placing dry penicillin in the center of the cold mold which had been covered by a layer of hardened cocoa butter. One or two drops of the melted oil closed the opening, forming the base of the suppository capsule which was then kept under refrigeration until immediately before administration (Table III).

Insufflation of penicillin was carried out through an anoscope into the rectum of five subjects (Table VI). A small amount of water-soluble lubricant was applied to the outside of the instrument before its introduction.

For penicillin assay as shown in Tables I, II, and V, the Food and Drug Administration cup plate method[16] was employed in the urine and a modification of Rammelkamp's method[17] in the serum.* In the latter determination, the susceptibility of the lyophilized hemolytic streptococcus strain used as the inoculum averaged 0.015 unit per milliliter.

Penicillin assay listed in all other tables (Tables III, IV, VI, and VII) was performed by means of (1) a modification of Fleming's micromethod[18]† and (2) a serial dilution method similar to Kolmer's[19] employing the *Staphylococcus aureus* strain 209 P as the test organism.‡ A number of specimens were subjected to parallel tests by either method, as indicated in the tables by two figures representing one specimen.

Streptomycin in the urine was determined according to the method of the Food and Drug Administration.[20]*

RESULTS

Absorption of penicillin from a suppository was prompt and rapid (Table I); the highest blood level in every case was found in the first blood specimen taken; the fifteen-minute level, whenever obtained, exceeded the half-hour reading which in turn was invariably higher than the result of the one-hour sample. Most significant in this connection was the occurrence (Case 5) of sudden diarrhea ten minutes after the suppository was inserted; the blood titer five minutes thereafter, as well as the penicillin excretion in the six-hour urine specimen, compared well with the respective findings in other trials in which no such loss was observed. Penicillin activity of the serum was still well assayable after six hours whenever determined in Cases 1 to 11, and after nine hours in Cases 8 and 11 (two subjects receiving high dosage). In all instances in which fractional urine samples were examined, the quantity of penicillin excreted in the first three hours exceeded the amount found in the following nine hours and amounted in most cases to more than 50 per cent of the twenty-four hour recovery. The latter ranged between 3.6 and 8.3 per cent, a mean percentage recovery of 6.3.

*These assays were carried out at the Venereal Disease Research Laboratories, U. S. Marine Hospital, Staten Island, N. Y.

†Carried out at the Department of Biochemistry, Northwestern University Medical School, Chicago, Ill. (Chief, Dr. C. J. Farmer.)

‡Unpublished method by Miss Helen MacLean, Department of Bacteriology, Michael Reese Hospital, Chicago, Ill.

TABLE I. SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING RECTAL ADMINISTRATION OF AMORPHOUS SODIUM PENICILLIN BY COCOA BUTTER SUPPOSITORY

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML.) HOURS					PERCENTAGE OF DOSE EXCRETED HOURS		
		¼	½	1	3	6	3	12	24
1	0.5			0.125	0.03	0.033 (7 hr.)	-	4.5	5.7
2a	0.5			0.107	0.025	0.018	-	7.4	7.5
3b	0.5	0.833	0.75	0.214	0.03		6.8	7.0	7.0
4c	0.5	0.214	0.187	0.075	0.025	0.025	3.6	4.2	4.2
5d	0.5	0.37					-	3.3 (6 hr.)	-
6	0.5		0.545	0.075			7.8	-	-
7	0.5		0.214	0.15	0.015		3.8	-	-
8*	1.0		0.5	0.187	0.107	0.093	-	5.2	8.3
9*	1.0			0.3 (1½ hr.)	0.042	0.015	-	6.3	7.4
10	1.0	0.75	0.6	0.15	0.05	0.042	2.3	-	4.5
11d*	1.0	1.25	0.75	0.3		0.107	2.5	4.9	5.4
12a	0.5 (2 hr. after streptomycin)		0.166	0.018	0	0	2.5	-	3.0
13c	0.5 (2 hr. after streptomycin)		0.125	0.08	0	0	1.7	2.7	3.1
14b	0.5 (2 hr. after streptomycin)	0.5	0.187	0.136	0.015	0	4.7	6.1	6.1
15	0.5 (2 hr. after streptomycin)	0.05	0.025	0	0	0	0.8	1.6	1.6

Letters a, b, c, and d indicate same patients subjected to different experiments.
0, Less than the minimal amount of penicillin inhibiting the test organism.
*Serum level at nine hours in Case 8, 0.093; Case 9, 0; Case 11d, 0.023.

The rectal application of streptomycin preceding the insertion of a penicillin suppository (Table I, Cases 12 to 15) caused an apparent lowering of penicillin values in both blood and urine. This fact is particularly obvious when the results in Cases 12 to 14 are compared with those obtained in the same patients without the use of streptomycin (Cases 2 to 4). After three hours, penicillin activity of the blood was at or below the sensitivity limit of the test organism.

In all urine specimens collected in Cases 12 to 15, the streptomycin content was either minute (Case 14) or not demonstrable, a result expected from investigations of oral ingestion.[21]

In the retention enema group (Table II), penicillin activity in the serum tested in each case in as many samples as in the suppository series was found to be either very low (maximum was 0.05 unit per milliliter at six hours in Case 4) or not assayable at all. Also the total urinary recovery was generally less in the saline group. The quantity of penicillin excreted during the first collection period was usually smaller than in the subsequent sample, in contrast to the predominantly inverse ratio in the former series.

The findings in cases treated with cocoa butter capsules (Table III) were, in part, similar to those experienced in the suppository trials, with early peaks of the blood levels and a penicillin excretion predominant within the first few

TABLE II. PENICILLIN EXCRETION IN URINE FOLLOWING ADMINISTRATION OF AMORPHOUS SODIUM PENICILLIN BY RECTAL MICROCLYSTER

CASE	DOSAGE (MILLION UNITS)	PERCENTAGE OF DOSE EXCRETED IN HOURS		
		3	12	24
1	0.1	1.6	2.5	2.5
2	0.5	0.3	1.0	1.1
3	0.5	0.5	1.5	1.5
4e	0.5	0.3	5.0	8.7
5e	1.0	0.2	0.8	1.2

e, Same patient subjected to different experiments.

TABLE III. SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING ITS RECTAL ADMINISTRATION BY COCOA BUTTER CAPSULE

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML.) IN HOURS			PERCENTAGE OF DOSE EXCRETED IN HOURS	
		¼	½	3	3	6-21
1	0.2 amorphous sodium		2.0*		24.8 23.8*	30.4
2	0.2 amorphous calcium	8.0*			21.5 32.5*	22.5
3	0.2 amorphous calcium	2.56 4.0*		0.08 0.25*	15.4	16.8
4†	0.2 crystalline sodium		1.28	0.08	25.6	26.2
5‡	0.5 crystalline sodium	12.8	6.4 (1 hour)	0.4	28.8	35.2
6§	0.1 amorphous sodium	2.56		0.04	14.5	–
7§	0.2 crystalline sodium G	5.12		0.16	17.2	18.1

*Results determined at Northwestern University.
†Defecation occurred ten minutes after insertion of capsule.
‡Seven-year-old boy weighing 56 pounds. Serum level at five hours, 0.32.
§Six-year-old boy weighing 45 pounds.

hours. The excretion ratio of 26.2 per cent in Case 4, where defecation occurred ten minutes after insertion of the capsule, deserves special attention. The greater height of the serum levels and the average urinary recovery ratio of about 25 per cent make this method far superior to the simple suppositories.

With the use of rectal gelatin capsules* (Tables IV and V), the urinary penicillin excretion was generally lower and within a much wider range, allowing no mean value to be computed. The serum concentrations likewise were lower than those shown in Table III. The half-hour readings in Cases 1 and 4 were higher than those of the earlier levels, and later urine specimens often contained more penicillin than the first three-hour collections, suggesting a relative delay in absorption.

In the insufflation experiments, the actual amount of penicillin reaching and adhering to the mucosa was surely less than the quantity obtained from the commercial vials, since diffusion of some of the powder over the inner surface of the instrument and over the examiner's face and clothing was noted following every such procedure. Therefore, the true absorption ratio for the penicillin which remained in the rectum is undoubtedly better than appears from Table VI. Still, most results here are comparable with those found with cocoa butter

*Gelatin capsules, Rectal No. 1, Parke, Davis & Company, Detroit, Mich.

TABLE IV. SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING ITS RECTAL ADMINISTRATION BY GELATIN CAPSULE

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML.) HOURS ¼	½	1	2	3	PERCENTAGE OF DOSE EXCRETED HOURS 3	15-24
1	0.2 amorphous calcium	0.08	1.28				5.3	14.0
2	0.2 amorphous calcium		0.25*			0*	2.3*	10.3*
3	0.2 amorphous calcium		0.4				9.2	13.5
4	0.5 crystalline sodium	2.56	5.12		0.8		18.0	22.7
		2.0*	4.0*		1.0*			
5†	0.2 crystalline sodium		0.5*	0.125*		0*	18.2	-
6†	0.5 crystalline sodium		3.2	0.4		0.08	-	-

*Results determined at Northwestern University.
0, Less than 0.06 which is the sensitivity limit of the test organism used.
†Same child as in Case 5, Table III.

TABLE V. PENICILLIN EXCRETION IN URINE FOLLOWING RECTAL ADMINISTRATION OF 0.5 MILLION UNITS OF CRYSTALLINE SODIUM PENICILLIN BY GELATIN CAPSULE

CASE	PERCENTAGE OF DOSE EXCRETED HOURS 3	5	24
1	3.7	4.8	7.2
2	1.2	4.3	5.1
3*	0.3	0.6	1.0
4	0.1	4.1	5.8
5	8.7	10.7	18.0

*Defecation occurred at the sixth hour.

TABLE VI. SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING RECTAL INSUFFLATION OF 0.2 MILLION UNITS OF AMORPHOUS CALCIUM PENICILLIN

CASE	SERUM LEVELS (UNITS PER ML.) HOURS ¼	½	3	PERCENTAGE OF DOSE EXCRETED HOURS 3	6-20	REMARKS
1		1.28		30.7*	34.5*	Fecal scybala visible in rectum
		1.0*				
2	>1.6		0.08	14.7	-	Insufflation done after liquid defecation; much backfire
	8.0*			22.1*	22.3*	
3	8.0*			33.3	-	Scybala visible in rectum
				49.9*		
4	2.0*			15.3*	17.4*	Marked diffusion
5	0.64		0	5.4	5.7	Some fecal matter visible in rectum; marked diffusion

*Results determined at Northwestern University.
0, Less than 0.04, the sensitivity limit of the test organism.

TABLE VII. SERUM LEVELS AND URINARY EXCRETION OF PENICILLIN FOLLOWING ADMINISTRATION OF CRYSTALLINE SODIUM PENICILLIN BY INTRAMUSCULAR INJECTION OF AN AQUEOUS SOLUTION AND BY INGESTION IN A GELATIN CAPSULE ONE-HALF HOUR BEFORE NOON MEAL

CASE	DOSAGE (MILLION UNITS)	SERUM LEVELS (UNITS PER ML.) HOURS ¼	½	3	PERCENTAGE OF DOSE EXCRETED HOURS 3	24
1	0.1 (intramuscularly)	10.24		0.16	69.2	70.9
		8.0*		0.25*		
2†	0.5 (orally)	2.56	1.6	0.08	39.4	43.8

*Results determined at Northwestern University.
†Same child as in Case 5, Table III.

capsules in regard to both speed and degree of systemic uptake. The presence of much fecal matter (Cases 1 and 3) did not seem to interfere noticeably with absorption.

DISCUSSION

Rectal absorption from a penicillin solution is more erratic and less prompt and efficient than from a suppository or cocoa butter capsule containing the drug. The reason for this could be passage of enemas into the proximal portions of the colon[22] with more limited absorption capacity and exposure of penicillin in the aqueous medium to bacterial penicillinase action. On the other hand, a suppository or capsule remains in the lower rectum just above the sphincters where absorption is enhanced by the hemorrhoidal venous plexus with its portal and caval communications[23]; besides, penicillinase activity is unlikely in an oily medium.[24] This distinction in absorption between penicillin suppositories and enemas appears both to confirm and explain the contradictory experimental reports referred to.

The rapid initial absorption from a simple suppository associated with a relatively low blood level and urinary excretion, when first observed, indicated a retention in the rectum of most of the drug. The early descent of the blood level was probably not due to penicillinase interference, since it takes several hours to record in in vitro emulsions of stools with penicillin a marked loss of antibiotic activity.[6] Moreover, the streptomycin suppositories given in order to combat colon bacillus activity and to increase penicillin absorption were found to depress the latter (Table I). These facts pointed to the accumulation in the rectum of the oily material and to its intimate contact with penicillin as important interfering factors. The experiments with insufflation and with cocoa butter capsules, containing the vehicle in smaller quantity and unmixed with the antibiotic, seem to have borne out these conclusions; the efficiency of absorption here is about fourfold.

The mostly disappointing results with gelatin capsules may be accounted for by slow or incomplete disintegration of gelatin and/or by possible adsorption of penicillin to the dissolved gelatin or to the lubricant used in some instances.

The insufflation experiments, inaccurate as they may be, suggest that absorption from pure dry penicillin applied directly to the rectal mucosa is prompt and therapeutically significant. No noteworthy local irritation was observed in these or in any other of the experiments reported.

The type of penicillin used, as indicated in the tables, does not seem to have had a noticeable bearing on the results.

The accuracy of all the findings tabulated, save for the saline group, is impaired by the inevitable loss of penicillin incurred in the preparation of the various suppositories and capsules, estimated at 5 to 10 per cent. Dosages as listed in the tables are, of course, copied from the labels of the vials from which the drug was obtained. The true serum and excretion values, therefore, in each such experiment must be regarded as somewhat higher than tabulated. Needless to say, the error inherent in the methods of bio-assay employed is considerable (±20 per cent).

In any case, the average excretion ratio of penicillin applied rectally either by insufflation or in cocoa butter capsules appears to amount to no less than one-fifth of the absorption coefficient of injected penicillin (Table VII), which ranges between 60 and 100 per cent.[5, 25] Thus the rectal route is not inferior to the oral route which produces an average penicillin absorption of 20 per cent.[6, 7, 26] This is also evident from a comparison of the results of oral administration (Table VII, Case 2) with those of rectal administration (Table III, Case 5) in the same child. With the other methods of application tested the absorption ratio is lower. The usual rapidity of absorption from the cocoa butter preparations seems to rule out any significant interference by bacterial action. Further trials with different menstrua and improvement in the manufacture of suppositories, it is hoped, will reveal an even greater efficiency of penicillin absorption from the rectum and make it a useful means of systemic administration.

SUMMARY

The absorption ratio of penicillin from the rectum is chiefly determined by the method of administration. It is very low after microclysters of penicillin solution, about 6 per cent in the case of cocoa butter suppositories, and subject to wide variations with gelatin capsules. Absorption of penicillin insufflated or applied in cocoa butter capsules into the rectum is equivalent to upper intestinal absorption. The clinical use of the rectal route seems to depend upon ultimate selection of the most suitable vehicle.

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PARENTERAL NUTRITION

III. Studies on the Tolerance of Dogs to Intravenous Administration of Fat Emulsions

Harvey S. Collins, M.D., Lisbeth M. Kraft, D.V.M., Thomas D. Kinney, M.D., Charles S. Davidson, M.D., John Young, M.D., and Fredrick J. Stare, M.D.
Boston, Mass.

PREVIOUS work in this laboratory has dealt with the problem of fat emulsions suitable for intravenous administration.[1-3] The desirability of fat as a part of parenteral nutrition is evident as a means of securing a high caloric intake with a minimum of fluid volume. With a sufficiently high caloric intake the utilization of protein from parenteral sources would be maximal, the destruction of body protein as a result of a caloric deficit would be minimal, and it ought to be possible to improve considerably the nutritional status of a severely emaciated child or adult. That emulsions given intravenously are utilized has been shown in the dog by gain in weight, by total carcass analysis for fat, and by change from negative to positive nitrogen balance by increasing only calories as a result of intravenous fat.[3]

In the studies previously reported, a 15 per cent fat emulsion was infused into dogs at a slow rate. Granulomatous lesions were found principally in the lungs and spleen, and to a less extent in the liver, after daily infusion for thirty to ninety days. The fat emulsion contained, in addition to fat, a soybean phosphatide preparation as a stabilizing agent; this material may have been primarily responsible for these histopathologic lesions. The purpose of the present study is to report observations on the following: the tolerance of dogs to emulsions of 30 per cent fat and to the rapid administration intravenously of such emulsions, the length of time infused fat remains in the blood, and whether the fat or the stabilizer is primarily responsible for the granulomatous lesions.

EXPERIMENTAL

A total of sixteen adult mongrel dogs was used in these studies. The fat emulsions were infused into the leg veins as usual. The preparation of the fat emulsions was similar to that previously used[2] and the composition is given in Table I. Total plasma fatty acids were determined by the method of Bloor[4]; the hematologic studies were made by the usual techniques; and the determinations of bromsulfalein elimination, plasma phosphatase, and plasma cholesterol components were as previously described.[5] In spite of an epidemic of distemper which developed while the experiments were in progress, the fat infusions were

From the Department of Nutrition, Harvard School of Public Health; the Departments of Biological Chemistry, Medicine, and Pathology, Harvard Medical School; the Department of Pathology, Peter Bent Brigham Hospital; and the Thorndike Memorial Laboratory, Boston City Hospital.

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given as planned. Much of the chemical work is not reported, nor the post-mortem examinations in all animals, because of the difficulty of interpretation resulting from the distemper.

TABLE I. COMPOSITION OF FAT EMULSIONS FOR INTRAVENOUS ADMINISTRATION

CONSTITUENTS	EMULSION			
	11M	12	13	14
Water (in ml.)	813	609	948	970
Refined coconut oil (in Gm.)	150	300	20	—
Soybean phosphatides* (in Gm.)	27	54	27	27
Na_2HPO_4 (in Gm.)	6.0	11.6	6.0	6.0

The composition given yields approximately one liter of each emulsion.
*Asolectin, Associated Concentrates Inc., Elmhurst, Long Island, N. Y.

Experiment 1.—The object of this experiment was to study the tolerance of dogs to a more concentrated fat emulsion than had previously been used (30 per cent fat versus 15 per cent) when given at a rapid rate. Nine dogs were divided into three groups of three animals each. A 30 per cent fat emulsion (Emulsion 12, Table I) was given intravenously so that two, four, and eight times as many grams of fat per kilo of body weight per minute were given as in previous studies using a 15 per cent fat emulsion. The data obtained from these dogs are shown in Table II. Three of the dogs (Dogs 51, 54, and 55)

TABLE II. RATE OF INTRAVENOUS INFUSION OF A 30 PER CENT FAT EMULSION (EMULSION 12) INTO DOGS IN EXPERIMENT 1

	GROUP I			GROUP II			GROUP III		
Dog	50	51*	52	53	54*	55*	47	48	49
Mean infusion time (min.)	21	20	21	41	40	40	77	77	75
Days infused (total)	20	8	20	20	16	13	18	21	21
Rate of infusion (Gm. fat/kg. of dog/minute)	0.13	0.13	0.13	0.066	0.066	0.066	0.036	0.035	0.031
Mean weight of dog (kg.)	14.5	14.8	14.8	12.0	13.0	14.5	13.7	11.4	19.7
Total fat infused (Gm.)	770	300	780	670	570	500	660	640	1040

*Dog died before completion of experiment.

died before the completion of the experiment. As far as could be ascertained from clinical symptoms and post-mortem examinations these deaths were due to distemper and to other intercurrent infection.

The amount of fat infused daily per kilo of body weight was the same in each group, averaging 2.6 Gm., but the infusion time approximated twenty-one minutes in the dogs of Group I, forty minutes in Group II, and seventy-seven minutes in Group III. In previous studies using a 15 per cent emulsion, the amount of fat infused daily varied from 1.7 to 2.8 Gm. per kilo and the infusion time was much longer, varying from one hundred thirty-eight to one hundred seventy-nine minutes. The animals appeared to tolerate the 30 per cent fat emulsions well, particularly after the first two or three days. During the first few days many of the animals vomited, but after this time no further vomiting was noted. To control the delivery of the 30 per cent emulsion through a No. 19 needle it was necessary to apply a slight positive pressure to the infusion bottle, especially for the infusions given in twenty-one minutes.

Dog 50 of Group I is of particular interest. The dog became distemperous despite prophylaxis with immune serum and lost 1.5 kg. prior to the onset of

infusions. The animal refused to eat; however, the course originally planned for this animal was pursued and infusions with the 30 per cent emulsions were commenced. It was hoped that the calories available in the fat emulsion might assist in preventing further weight loss resulting from the distemper. The emulsion was infused for twenty consecutive days at a very rapid rate (0.13 Gm. fat per kilo of dog per minute). The weight curve of this animal is given in Fig. 1 and it is seen that by the tenth day of infusion the body weight had fallen to its lowest level, 12.9 kg., from 15.9 kilograms. Virtually no oral nutrients had been taken by the dog during the preceding sixteen days which represented the clinical period of acute distemper. At this point the clinical condi-

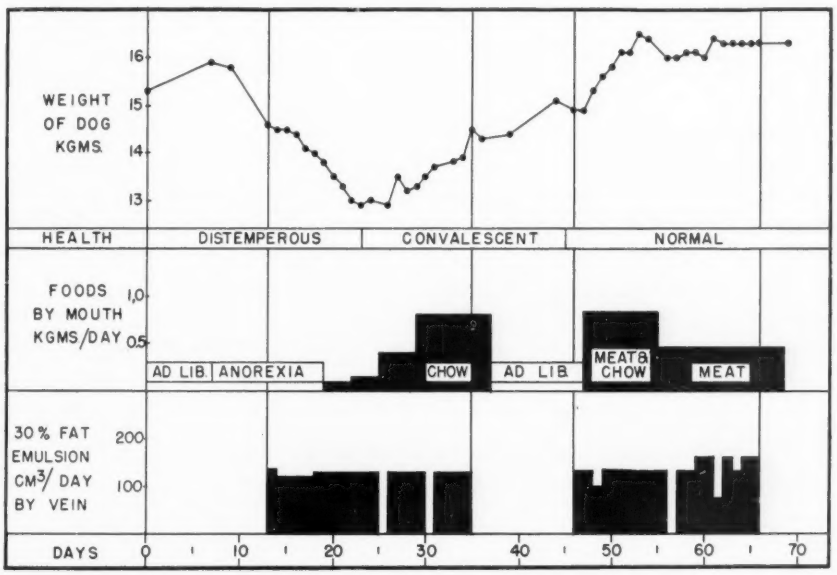

Fig. 1.—Intravenous administration of 30 per cent fat emulsion to Dog 50, Experiment 1.

tion of the dog began to improve and appetite returned and in a few days was brisk. Chow* and horse meat were fed ad libitum and the daily infusions of fat were continued. At the termination of the twenty-day infusion period the dog weighed 14.5 kg. and appeared healthy. It was decided not to sacrifice Dog 50 at this time, but to give another series of fat infusions so that the effect of the 30 per cent emulsions given rapidly could be observed on the weight and clinical condition of the animal when it was healthy and free of distemper. An interval of eleven days elapsed between the two periods of fat infusions (see Fig. 1), during which time the dog was fed chow and horse meat ration in an amount such that gain in body weight approximated 0.5 kg. throughout the eleven-day interval. These same average daily intakes of oral nutrients were continued for the first eight days of the second infusion experiment, but with the rapid gain in weight, which must have resulted from the intravenous fat, all chow was omitted and only the horse meat in amounts previously given was allowed for the last twelve days of the experiment on fat infusions. No

*Furnished through the courtesy of the Research and Development Department, General Foods Corporation, Hoboken, N. J.

weight loss occurred during this second period of fat infusion which was similar to the first period in the emulsion used, the total amount of fat given, the rate at which it was given, and the number of days the animal was infused. Indeed, there was a gain in weight of 1.4 kilograms. The dog appeared perfectly normal at the end of the experiment. Bromsulfalein tests done periodically throughout the complete experiment (sixty-six days, see Fig. 1) suggested at times a moderate impairment of liver function as previously noted,[3] but results frequently were normal.

At post-mortem examination this animal (Dog 50) showed granulomatous lesions in the lungs which were similar to those described previously.[2, 3] These consisted of small nodules of two or three giant cells surrounded by round cells. There were also several larger collections of round cells and polymorphonuclear leucocytes that were not associated with giant cells. Small collections of macrophages with yellow pigment were in the alveoli. Large collections of polymorphonuclear leucocytes were seen in the portal areas of the liver but without granulomatous lesions or fatty metamorphosis. There were collections of round cells in the kidney and several large nodules of lymphoid and plasma cells and fibroblasts. No giant cells were observed. The heart, spleen, gastrointestinal tract, pancreas, adrenals, lymph nodes, blood vessels, and brain were normal.

Sections of the lungs of the other dogs of Experiment 1 that were alive at the completion of the experiment showed approximately four relatively small granulomatous lesions per low-power field. The livers of Dogs 52 and 48 showed polymorphonuclear leucocytes in the portal areas. There was no fatty metamorphosis of the liver in any of the animals. There was yellowish-brown pigment in the spleen and lymph nodes. The other organs were normal.

Experiment 2.—The object of this experiment was to determine whether the fat or the phosphatide was principally responsible for the granulomatous lesions that had been observed following infusion of fat emulsions. In this experiment a total of six dogs was used. They were divided into three groups of two each and for the first two weeks of the study were fed horse meat and dog chow ad libitum though food consumption records were kept. During the next two weeks the amount of horse meat and dog chow fed daily was arbitrarily cut to half the average daily amount of the first two-week period. At the beginning of the third period (twenty-ninth day of experiment) all the dogs received intravenous infusions in addition to the amount of horse meat and dog chow given in the second period with the reduced rations. One group received Emulsion 11M (15 per cent fat); the second group, Emulsion 13 (2 per cent fat); and the third group, Emulsion 14 (no added fat). These infusions were given daily for the next thirty-two days in the amount of 12 to 14 ml. per kilogram body weight per day and in a period of twenty minutes. The experiment was terminated on the sixty-first day for one of the dogs receiving the 15 per cent emulsion and for both of the dogs receiving the 2 per cent emulsion. The remaining three animals were infused for forty more days, though the two dogs that had received Emulsion 14 without any added fat were permitted ad libitum oral feeding. Since the response in weight for the animals in each group was similar, only one weight curve for each group is given; these are in Fig. 2. Hematologic and chemical data obtained on these dogs are given in Table III.

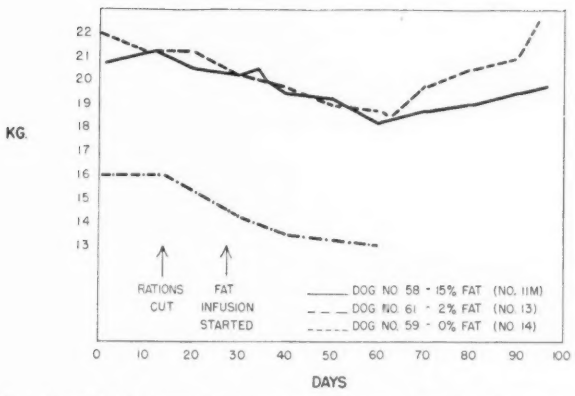

Fig. 2.—Weight curves of representative dogs in Experiment 2.

TABLE III. HEMATOLOGIC AND CHEMICAL DATA OBTAINED ON DOGS IN EXERIMENT 2

	Dog 58							*Dog 60*						
Weeks of experiment	1	3	5	7	9	10	13	1	3	5	7	9	10	13
15% Fat emulsion 11M, given I.V. as indicated by bar														
Hemoglobin (Gm. per 100 c.c.)	13.5	13.5	11.8		12.0		10.9	15.1	14.4	13.4		13.1	12.7	
Hematocrit (%)	40.6	38.8	34.5		35.9		32.8	45.1	40.9	40.5		38.3	38.7	
Reticulocytes (%)	0.5	0.0	0.0		0.0		0.0	0.2	0.8	0.0		0.0	0.2	
Bromsulfalein test	6.0	6.0	11		12.0		8.0	10	10	12		10	12	
Plasma total cholesterol		61	128		126		100	123	129	142		140	153	
Plasma cholesterol esters		42	86		70		72	85	88	56		75	89	
Total plasma fatty acids		452	453		369		400		222	393		310	347	
Plasma phosphatase		55	100		150		195		169	190		300	354	

	Dog 61							*Dog 64*						
Weeks of experiment	1	3	5	7	9	10	13	1	3	5	7	9	10	13
2% Fat emulsion 13, given I.V. as indicated by bar														
Hemoglobin (Gm. per 100 c.c.)	14.0	14.5	14.0		14.4			14.8	16.6	16.6	14.2	13.3		
Hematocrit (%)	39.9	44	43.8		43.6			44.3	48	48.6	42.3	40.8		
Reticulocytes (%)	0.1	0.0	0.2		0.0			0.0	0.2	0.8	0.0	0.0		
Bromsulfalein test		4	8		6			10	10	12	10	12		
Plasma total cholesterol		153	236		179			130	107	182	200	173		
Plasma cholesterol esters		103	167		106			85	69	91	110	115		
Total plasma fatty acids		222	189		179			260	303	318	180	302		
Plasma phosphatase		150	321		333			37	67	204	363	350		

	Dog 59							*Dog 63*						
Weeks of experiment	1	3	5	7	9	10	13	1	3	5	7	9	10	13
No fat, only phosphatides (Emulsion 14) given I.V. as indicated by bar														
Hemoglobin (Gm. per 100 c.c.)	16.2	17.8	17.5	16.5	13.5	14.1	13.8	14.5	14.5	13.5	14.7		13.4	14.2
Hematocrit (%)	53.6	49.2	50.3	49.2	40.0	43.1	41.1	45.5	45.1	40.0	43.4		43.6	47.2
Reticulocytes (%)	0.0	0.1	0.0	0.0	0.4		0.0	0.2	0.0	0.0	0.0		0.3	0.0
Bromsulfalein test	12	10	10	8	12	10	8		6	4	4		7	10
Plasma total cholesterol	131	163	149	160	175	197	146		110	100	77		130	140
Plasma cholesterol esters	72	100	59	75	90	66	80		90	69	35		72	80
Total plasma fatty acids		460	296	340	300	203	404		440	172	423		320	325
Plasma phosphatase		90	162	200	175	154	355							

Plasma phosphatase, micrograms of inorganic phosphorus liberated per cubic centimeter of plasma in twenty-four hours at pH 7.6.

Bromsulfalein test, micrograms of dye per cubic centimeter of plasma at eight minutes.

Plasma total cholesterol, milligrams per cent.

Plasma cholesterol esters, milligrams per cent.

Total plasma fatty acids, milligrams per cent.

Fat tolerance curves: At various times throughout the fat infusion periods of the two dogs receiving the 15 per cent fat emulsion (Dogs 58 and 50) in Experiment 2, a number of fat tolerance studies were made. These consisted of determining the total plasma fatty acids immediately before the infusion and at varying intervals during and after. Two typical curves are shown in Fig. 3.

Post-mortem examination: All the dogs of Experiment 2 were autopsied at the termination of the experiment. The dogs that received either the 15 per cent or the 2 per cent fat emulsion showed essentially similar pathologic changes.

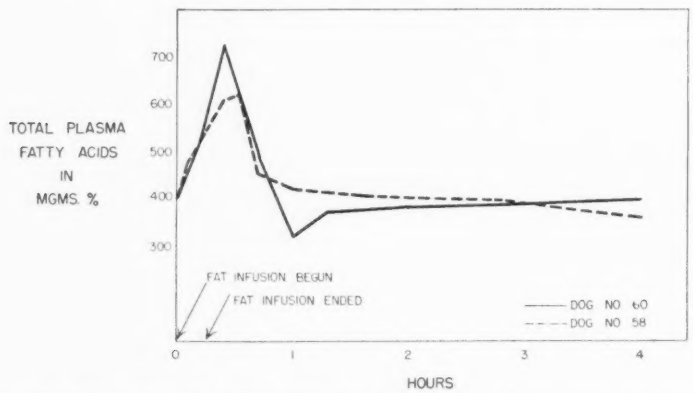

Fig. 3.—Fat tolerance curves in dogs following infusion of 15 per cent fat Emulsion 11 M in Experiment 2.

Granulomatous lesions in the lungs were fewer and less severe than those found in the lungs of the animals in Experiment 1 and also less severe and less numerous than those found in any of the animals which we have previously studied. All of the four animals had evidence of distemper. No granulomatous lesions were found in any of the other organs.

Dogs 59 and 63 given the soy bean phosphatide alone, are of particular interest. More severe and more numerous granulomatous lesions were found in the lungs of these dogs. Foreign body giant cells were strikingly prominent in these lesions and particulate matter was identified in many of these cells.

Experiment 3.—In this experiment a pregnant dog was given daily fat infusions. The dog was fed ad libitum a purified low fat ration (compare with ration 2, reference 3) during the first two-week period. Food consumption records were kept and during the second two-week period half the average daily consumption of the first period was given. At the beginning of the third period (twenty-ninth day of the experiment) daily fat infusions with Emulsion 11M (15 per cent fat) were started in addition to continuing the purified oral ration in the amount used in the preceding two-week period. An arbitrary amount of 300 ml. of fat emulsion was given daily. This furnished 460 calories, or an average of 33 calories per kilo of body weight. The quantity of purified ration fed amounted to 80 Gm. per day and furnished 309 calories, or an average of 22 per kilo of body weight. The fat infusions and purified ration were continued daily until the day the pups were born. They were not given on the day of delivery

or the day following, but then were given as previously for a further period of thirty-five days after which they were discontinued and ad libitum feeding of dog chow and horse meat continued for the rest of the experiment. On the day of delivery, milk, meat, and dog chow were fed ad libitum. A lobectomy was

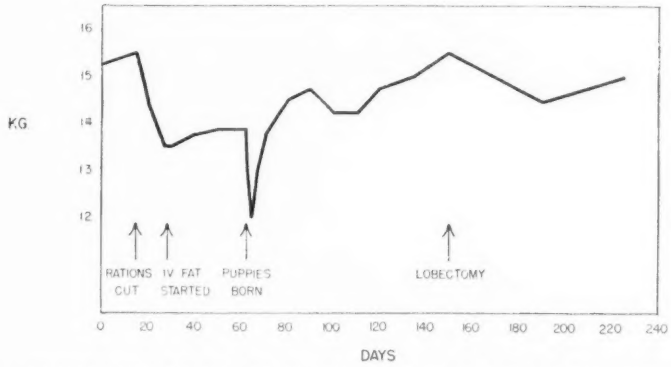

Fig. 4.—Weight curve for Dog 56, Experiment 3, fat infusion during pregnancy.

done on this animal eighty-eight days after delivery, which was fifty-two days after the last fat infusion. Weight and hematologic records were kept for an additional seventy-five days after which the animal was sacrificed for post-mortem examinations. The weight curve of this animal is given in Fig. 4 and the chemical and hematologic data in Table IV.

TABLE IV. HEMATOLOGIC AND CHEMICAL DATA OBTAINED ON DOG 56 IN EXPERIMENT 3, FAT INFUSION DURING PREGNANCY

Week of experiment	1	3	4	6	7	8	11*	19†	25	31
15% Fat emulsion 11M. given I.V. as indicated by bar										
Hemoglobin (Gm. per 100 c.c.)	15.6	14.9	14.0	11.6	9.7	9.7	9.4	13.1	15.9	16.1
Hematocrit (%)	50.7	48.7	42.2	32.2	29.8	29.9	30.9	39.6	51.8	49.2
Reticulocytes (%)	0.0	0.0	0.0	0.0	0.0	0.0	0.0			
Bromsulfalein	16	23		13		26	12			
Plasma total cholesterol	100	93		241		416	116			
Plasma cholesterol esters	73	80		124		160	69			
Total plasma fatty acids		384		309		578	584			
Plasma phosphatase		84		106		463	251			

*Two pups born.

†Lobectomy.

Plasma phosphatase, micrograms of inorganic phosphorus liberated per cubic centimeter of plasma in twenty-four hours at pH 7.6.

Bromsulfalein test, micrograms of dye per cubic centimeter of plasma at eight minutes.

Plasma total cholesterol, milligrams per cent.

Plasma cholesterol esters, milligrams per cent.

Total plasma fatty acids, milligrams per cent.

Post-mortem examination of this dog revealed no unusual findings grossly. Microscopic examination of the lobe of the lung removed surgically showed approximately one granulomatous lesion per two low-power fields. The lesions were smaller and less numerous than those generally found. The lungs at autopsy showed even fewer granulomatous lesions, approximately one per ten low-power fields. These lesions were even smaller than those seen in the lobe removed sur-

gically and there was very little scar formation. There was a fairly large area in the myocardium infiltrated with round cells and polymorphonuclear leucocytes. The liver showed focal areas of necrosis in the portal areas but no granulomatous lesions or fatty metamorphosis. There was hyperplasia of both the red and white series of the bone marrow. The spleen, gastrointestinal tract, pancreas, adrenal, lymph nodes, blood vessels, and brain were normal.

The autopsy on this dog was performed seventy-five days following the surgical removal of the lung and one hundred twenty-seven days after the last injection of fat. It is apparent from these findings that the lesions described tended to regress slowly, for there was a striking difference in the number and size of the lesions in the lung removed surgically and the lungs examined at the time of autopsy.

DISCUSSION

The studies in Experiment 1 demonstrate that fat can be given in an emulsion at least twice as concentrated as we had previously used (30 per cent as compared with 15 per cent) and at a much more rapid rate. These observations are of importance in the potential clinical use of fat emulsions because they indicate that a considerable number of calories in a limited fluid volume may be given in a relatively short time, thus eliminating a considerable increase in blood volume and prolonged periods of intravenous infusion.

The 30 per cent fat emulsion furnishes approximately 3.0 calories per milliliter. The dogs tolerated the emulsion well except for the first two or three days of the infusion period when there was considerable vomiting, but even this generally could be controlled if the emulsion was given slowly, especially during the first half of the infusion period.

Dog 50 of Experiment 1 is of much interest because it had two periods of fat infusions, the total amount of fat being the same in each period and quite large, namely 770 grams. The first of these infusion periods began a few days after the dog became distemperous and for a time the infused fat constituted the sole source of calories because the dog was completely anorectic. During this period the dog rapidly lost weight, but probably less than would have been experienced had the animal not been receiving fat. During the second infusion period, identical to the first except that the dog had recovered from distemper, the fat was well tolerated, weight gain was rapid, and the dog was in excellent condition at the end of the experiment.

In Experiment 2 a preliminary attempt was made to find out whether the fat or the phosphatide used as the stabilizing agent was principally responsible for the granulomatous lesions that had always been observed following infusion. For this reason, infusions of a 15 per cent fat emulsion, a 2 per cent fat emulsion, and an emulsion containing no added fat but simply the phosphatide preparation were made. Two dogs were given each preparation for varying periods of time as indicated in Table III. The histopathology produced in all of these animals was essentially the same but was more accentuated in the two dogs receiving the phosphatide alone. It appears that the phosphatide preparation is the basic cause of the granulomatous lesions we have observed.

The hematologic and chemical data obtained in the six dogs in Experiment 2 (Table III) reveal a consistent decline in the hemoglobin and hematocrit throughout the course of the infusions. With the exception of Dog 63, which was one of the animals receiving only the phosphatides, this change was essentially the same in all animals and was not dependent on the amount of fat in the emulsion. In none of the dogs was there any significant reticulocytosis suggesting that the anemia was not hemolytic. Bromsulfalein elimination in all dogs and plasma total cholesterol and cholesterol esters varied from normal to values somewhat higher. Plasma phosphatase values showed a definite increase throughout the infusion periods in all dogs except Dog 63.

Total plasma fatty acids values in the dog generally range from 200 to 450 mg. per cent, depending principally on the diet and the time interval after eating that blood is taken for analysis. As the fat tolerance curves in Fig. 3 show, there is a rapid rise in plasma fat following infusion of a fat emulsion to a value approximately twice the normal. As soon as the infusion is finished, the plasma fat begins to decrease and in the short time of approximately one hour is back to normal. It is evident that infused fat leaves the blood stream rapidly.

While Experiment 3 consists of observations on only one dog, it is of particular interest in that it presents the results of fat infusion in a dog throughout the last half of pregnancy. The average oral intake of this animal was decreased during the second two weeks of the experiment to one-half the amount consumed during the first two-week period. From Fig. 3 it is seen that during the second two-week period there was a progressive weight loss totaling 2 kilograms. This weight loss was stopped at the end of the second two-week period when infusions with the 15 per cent fat emulsion were started. These infusions furnished an additional 460 calories per day, or 33 calories per kilo of body weight. The infusions were given daily, beginning with the twenty-ninth experimental day, except on the day of whelping and the day immediately following. They were discontinued on the ninety-eighth experimental day. There were only two pups in the litter and they were small and scrawny; however, both lived and developed into healthy dogs. The mother had no difficulty in feeding them.

A lobectomy was done fifty-two days after the last of 120 daily fat infusions. The animal was sacrificed seventy-four days following the lobectomy which was the two hundred and twenty-fourth experimental day. At the end of the experiment, the animal had completely made up all weight loss. Hemoglobin and hematocrit values at the end of the study were essentially the same as at the beginning, though throughout the course of the experiment both had fallen, due no doubt to the combined effects of pregnancy and the daily infusions. Bromsulfalein clearance, cholesterol values (both total and esterified), plasma fat, and plasma phosphatase remained essentially normal except for the values obtained just prior to whelping when they were all increased as is shown in Fig. 3.

SUMMARY

1. A 30 per cent fat emulsion stabilized with soybean phosphatides (Asolectin) was given intravenously to dogs with the same ease as the 15 per cent emulsions used in our previous studies.

2. Fat emulsions can be given relatively rapidly to adult dogs.

3. Fat infused into the blood stream caused a prompt and marked increase in plasma fat but normal values were approached in the adult dog within an hour after the termination of the infusion.

4. It appears that the soybean phosphatide used as the stabilizer was primarily responsible for the granulomatous lesions we have observed following the use of intravenous fat emulsions.

We desire to express our appreciation to Miss Virginia Kent and to Miss Mary Maloney for technical assistance.

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PARENTERAL NUTRITION

IV. Improved Techniques for the Preparation of Fat Emulsions for Intravenous Nutrition

Robert P. Geyer, Ph.D., George V. Mann, M.D., and Fredrick J. Stare, M.D.
Boston, Mass.

IN PREVIOUS reports from this laboratory[1-4] the use of fat emulsions for the intravenous nutrition of dogs has been described. One phase of the continuation of this problem has been the improvement of techniques for preparing emulsions of suitable particle size and stability. Since the relationship of particle size to such problems as fat embolism and fat utilization is not clearly understood at present, it seems desirable that *all* particles in such emulsions be no larger than normal chylomicra. When particles of this magnitude are dealt with, an accurate method of measuring particle size becomes of importance. In addition, there are the usual problems met with in intravenous nutrition, that is the elimination of pyrogens, sterilization, and the prevention of pathologic changes resulting from the administration of the material under study. The present paper deals with a photomicrographic method for determining particle size, a method for preparing fat emulsions whose maximum particle size is under 2 μ in diameter, and some of the preliminary studies on the physical stability of these emulsions.

EXPERIMENTAL

Photomicrographic Method for Determining the Size of Fat Particles.—Quantitative measurements of fat particles below 5 μ are difficult because of Brownian movement and low optical density. Since visual determinations are only approximate, a photomicrographic method was developed. In preliminary work both a tungsten filament and a carbon arc lamp were used as light sources, but were not found satisfactory at high shutter speeds. Subsequently a high speed, high-intensity, discharge lamp was found to be adequate. The apparatus is shown in Fig. 1. An Eastman precision enlarger No. 1 with a 35 mm. film roll adapter and holder was used without a shutter or lens, but was provided with a bellows adapter for the microscope. A Spencer research model microscope with an apochromatic lens system was used. Eastman Kodak "Plus X" 35 mm. roll film is satisfactory and considerably more convenient than Kodak "M plates" of Type B panchromatic sensitivity.

The light source consisted of an Edgerton type, high-voltage discharge lamp housed in a Spencer model 370 microscope lamp.* The lamp had a discharge

From the Department of Nutrition, Harvard School of Public Health, and Department of Biological Chemistry, Harvard Medical School.

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*Monarch Electrical Company, Lynn, Mass.

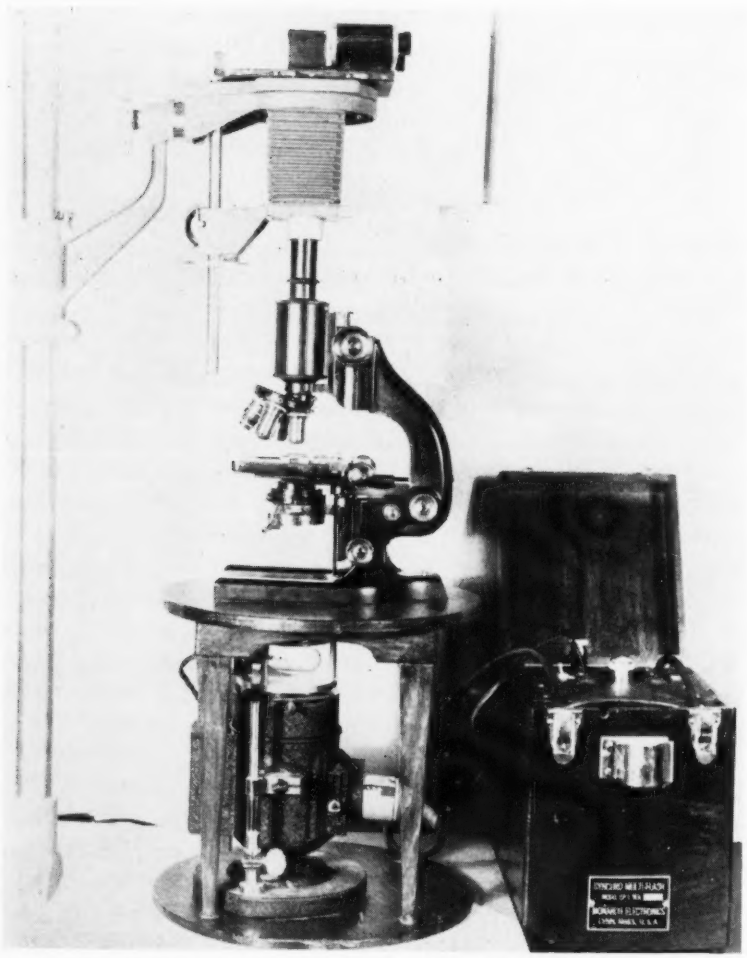

Fig. 1.—A photograph of the equipment used in preparing photomicrographs of emulsions.

time of $\frac{1}{50{,}000}$ second. Focusing of the microscope was accomplished by means of a General Electric 100 watt projection type tungsten filament bulb contained in the aluminum holder shown in Fig. 1. The focusing lamp assembly was previously centered with respect to the optical axis of the flash lamp. After focusing, the holder was removed from its position atop the discharge lamp casing.

In practice, a thin sample of undiluted emulsion was placed between a slide and cover slip and the chamber sealed with paraffin to prevent evaporation. For examination of a preparation the sample was removed from the surface, since the largest particles in the preparation tend to accumulate there. For some purposes sampling with a pipette and bulb at varying depths from the surface was done.

Calibration was accomplished by photographing and enlarging a slide micrometer in the same manner as was used for the emulsions. Measurements were then made by means of rule and caliper.

By a series of trials it was possible to establish the proper lamp diaphragm, field stop, and bellows length settings which would produce optimum results. Since the intensity and duration of the illuminating source were constant, the field stop on the lamp was used to control intensity. The substage condenser diaphragm was used to develop contrast. In general, maximal contrast was developed with the condenser diaphragm aperture reduced considerably below the numerical aperture rating of the objective used.

The combination of lens systems used was determined by the dimensional range of the material to be measured. For the emulsion particles under 2 μ the 2 mm. objective with oil immersions above and below the slide and with a 10X ocular gave the best results. A Wratten B filter was interposed between the ocular and the camera as a means of further accentuating contrast. This filter transmits between 480 and 620 millimicra. The bellows length was kept short. Additional magnification was accomplished when necessary by enlargement of the negatives.

For measurement, 3 by 5 inch enlargements of the negatives were satisfactory. Development of the negatives was designed to produce maximal contrast, and prints were made on No. 4 (Eastman) high-contrast paper.

In practice, each preparation measured is searched under high dry magnification for fields showing the largest particles and several of these are photographed, because maximal particle size is of prime importance. As mentioned below, in these emulsions the vast majority of the fat particles are less than 0.2 to 0.5 μ in diameter and thus beyond the resolution of the light microscope.

Fig. 2 illustrates representative photographs of three emulsions and the scale photographs used in the measurements.

Preparation of Emulsions.—Several different methods of preparing fat emulsions were studied under similar conditions to determine which warranted extensive consideration. Emulsions were produced by means of (1) a Waring blendor, (2) a hand homogenizer, (3) an ultrasonic generator,* and (4) a high-pressure homogenizer.† Aqueous dilution of a nonaqueous solution of fat and stabilizer was also tested. In each case the following constituents were used: coconut oil, soybean phosphatides,‡ and distilled water. Since the high-pressure homogenization method yielded the best emulsion from the standpoint of particle size, further studies were conducted using this method of production.

After preliminary work, the following general procedure was adopted for carrying out an emulsification.

Premixing of Emulsion Constituents.—Double distilled water previously boiled for four minutes was placed in a Waring blendor with the fractionated phosphatide preparation.§ The mixture was spun at top speed for two min-

*We are grateful to Dr. Ralph F. Shropshire and Mr. Edward W. Smith of the Submarine Signal Corp., Boston, Mass., for the use of their apparatus and for their assistance and cooperation.

†Junior Viscolizer 50, Cherry-Burrell Corporation, Charlestown, Mass.

‡Asolectin, supplied through the courtesy of Dr. Albert Scharf, Associated Concentrates, Inc., Elmhurst, Long Island, N. Y.

§This was prepared from commercial soybean phosphatides according to the procedure given in the following paper.

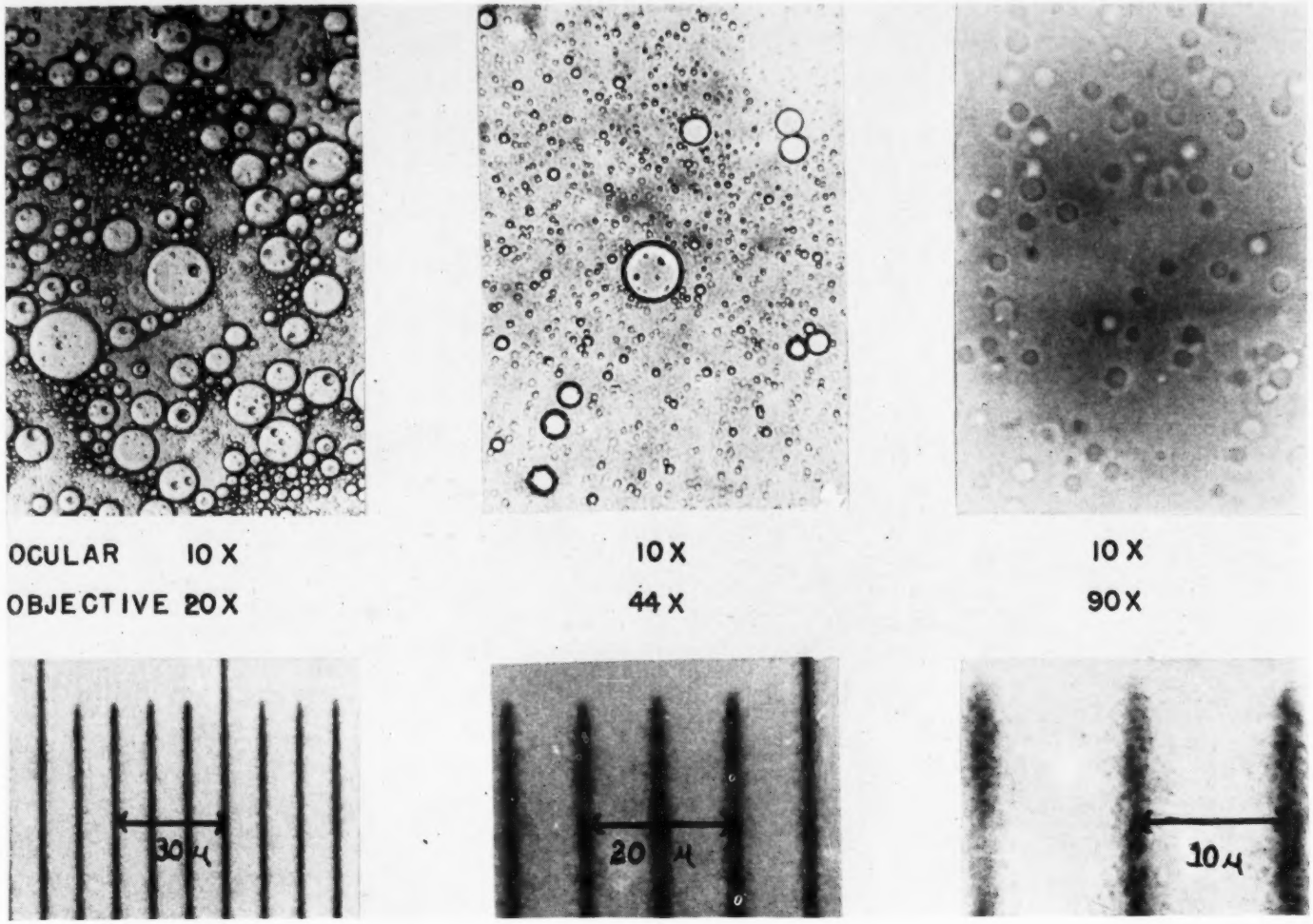

Fig. 2.—Photomicrographs of fat emulsions and micrometer scales for measurement. Representative photomicrographs made at the three most useful magnifications are shown. *A*, A broken emulsion photographed at low power; *B*, a relatively coarse emulsion photographed with the high dry lens system of the microscope; *C*, a fine emulsion photographed with oil immersion. In practice, the final magnification is determined by the bellows length of the camera and the degree of enlargement of the negatives made when printing.

utes and hot coconut oil (80 to 95° C.) was slowly added while the blendor was still rotating. Blending was continued for an additional three to five minutes. Throughout the entire process a flowing atmosphere of nitrogen was provided by means of a suitable attachment tube on the cover. Though the fat particles ranged from 1 to 15 μ in diameter, this degree of dispersion was sufficient temporarily to prevent excessive "oiling out" when the material was placed in the high-pressure homogenizer.

High-Pressure Homogenization Step.—The premixed material was introduced into the operating high-pressure homogenizer which was equipped with the external glass recyclizing system shown in Fig. 3. The homogenizer was a smaller model of the machine used by McKibbin and co-workers[2] and was better adapted for small volumes of material. Prior to the addition of the blended emulsion, the machine was freed of water by operating with the pressure regu-

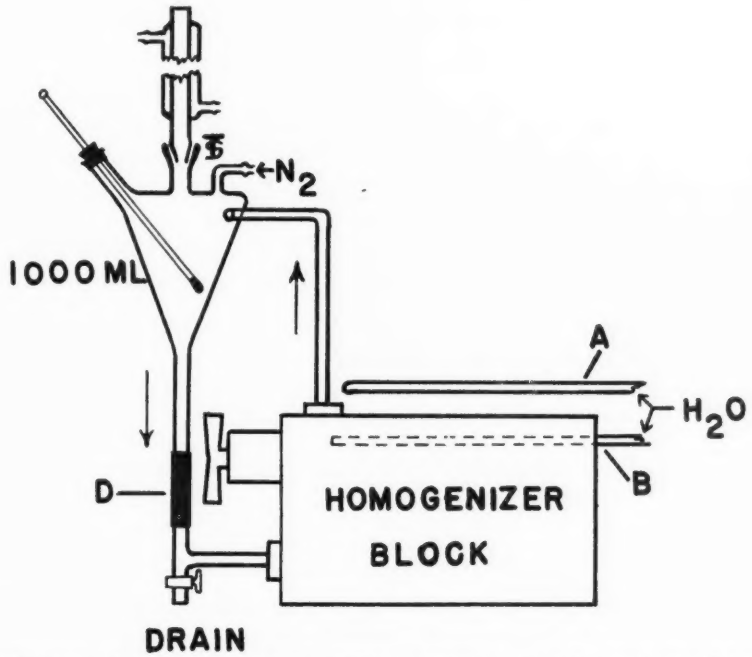

Fig. 3.—Diagram of high-pressure homogenizing apparatus used in preparing fat emulsions for intravenous nutrition.

lator block and the extra intake port opened. By means of a long, stiff wire bent in a right angle near one end, the suction valves were lifted to allow the water in the middle chamber to drain. After all parts were again replaced and tightened the machine was run while nitrogen was supplied to displace the air. When the blended material had been added, the thermometer was put in place and the flow of nitrogen was adjusted to a very slow rate. Where required, heat was supplied by regulating the flow of hot water (76° C.) through tube A (Fig. 3). In those cases where a low temperature was desired, the temperature of this water was lowered. In operation the condenser water flowed slowly

three most useful magnifications are shown. A, A broken emulsion photographed at low power; B, a relatively coarse emulsion photographed with the high dry lens system of the microscope; C, a fine emulsion photographed with oil immersion. In practice, the final magnification is determined by the bellows length of the camera and the degree of enlargement of the negatives made when printing.

over the piston rods through an appropriately placed three-holed length of copper tubing (*B*). This made it unnecessary to use the cooling system provided by the manufacturer and insured better control of the temperature of the equipment and homogenate. To remove trapped gas, the machine was run at zero pressure and the rubber tube (*D*) was alternately closed and opened. If this was unsuccessful the pressure was raised to 2,200 pounds and maintained until the temperature of the circulating homogenate reached 75 to 78° C., at which point the gas either escaped or could be removed by closing the rubber tube.

Preliminary investigation demonstrated that although many particles below 1 μ were formed by single passage of the material through the homogenizer, some particles as large as 6 to 8 μ remained even after ten to fifteen passages at pressure between 2,000 and 4,000 pounds per square inch. For this reason the material was continuously recyclized through the apparatus for periods of time ranging up to ninety minutes.

To determine the effect of temperature upon the rate of emulsification a series of tests were made using Emulsion 22 at 41, 60, 76, and 85° C. (For composition of Emulsion 22 see Table II.) The premixing in each case was done as described previously. It was found necessary to run cold water over the block to maintain the lowest temperature. Samples were removed at ten, twenty, and thirty-five minutes, and the size of the largest particles was ascertained. Table I contains the results of these tests carried out at a pressure of 3,000 pounds per square inch.

TABLE I. EFFECT OF TEMPERATURE ON THE RATE OF EMULSIFICATION
(VOLUME, 500 C.C.; PRESSURE, 3,000 POUNDS PER SQUARE INCH)

EXPERIMENT	TEMPERATURE (°C.)	APPROXIMATE DIAMETER IN MICRA OF LARGEST PARTICLES OF A 30% FAT EMULSION (EMULSION 22)		
		10 MIN.	20 MIN.	35 MIN.
1	41	8–10	4–6	<3
2	60	5–7	4–6	<3
3	76	4–6	2–4	<2
4	85	4–6	3	<1

The effect of pressure was studied by preparing 500 c.c. quantities of Emulsion 31 at 2000, 2500, 3000, 3500, 4000, and 4500 pounds per square inch respectively. (For composition of Emulsion 31 see Table II.) Homogenization was carried out for thirty to forty-five minutes at 76 to 80° C. Visual microscopic examination of samples removed at ten-minute intervals was used as a criterion. It was found that although all pressures used resulted in particles below 2 μ, the pressures above 3,000 pounds per square inch resulted in a greater proportion of the particles being at the extreme end of microscopic resolution. Thus emulsions prepared at higher pressures could be viewed microscopically when undiluted, without having the field of vision limited to the upper layers of the preparation. The emulsions appeared blue by reflected light in thin films and appeared red by transmitted light. These phenomena were observed with emulsions containing both 15 and 30 per cent of fat.

A number of emulsions were prepared in an investigation of the levels and ratios of fat and phosphatide conducive to small particle formation and stability. The amount of phosphatide was varied between 0.15 and 6.0 Gm. per 100 c.c., and the fat, between 10 and 30 Gm. per 100 cubic centimeters. The fat was either coconut oil, corn oil, or butter fat.* The emulsions prepared and the data pertaining to the conditions of preparation are given in Table II. Also included are the size of the particles produced and the effect of autoclaving for fifteen minutes at 15 pounds per square inch.

Fat emulsions are adversely affected by high electrolyte concentration, low pH (below 6.0), prolonged heating, evaporation of the water phase, and the presence of materials which carry electric charges dissimilar to those of the fat particles. Many of the emulsions given in Table II were studied under these adverse conditions. These studies are still in progress and will be reported in full at a later date. It is clear that for a high fat level more emulsifier is required for stability under adverse conditions. In addition it has been found that, with a given level of emulsifier, as the concentration of oil increases the emulsions are formed with increasing difficulty. It also has been found that small particles are of prime importance to stability. Thus, emulsions whose particles of fat are less than 2 μ in diameter and have a probable mean diameter below 0.5 μ are completely stable to autoclaving; whereas, otherwise comparable emulsions containing many particles above 5 μ either break or cream out under the treatment or upon standing.

Emulsions similar to Emulsions 22 and 31 have been used for extensive animal investigation because they are stable and are compatible with blood by in vitro and in vivo studies. However, as described in the following paper, the soybean phosphatide preparation has been fractionated so as to give a more desirable stabilizing fraction, and this fraction (BF2) is used in place of the Asolectin. In routine practice the emulsions are autoclaved in gas-tight bottles filled with nitrogen and with an aqueous dextrose concentration of 5 per cent. The bottles are stored in the dark at 24° C., and before intravenous use 1 c.c. of sterile 10 per cent Na_2HPO_4 is added to each 100 c.c. of emulsion. The pH of the emulsion is thus brought to 7.4. The results of the in vivo studies with these emulsions will be reported at a later date.

DISCUSSION

The photomicrographic technique has furnished a means of objectively evaluating emulsion preparations. It should be emphasized that the maximum particle size is the most important single factor in determining physical stability and may have more physiologic significance than simply assuring passage through the capillaries without embolization.

Under the proper conditions high-pressure homogenization resulted in the formation of fat emulsions whose largest particles were below 2 μ in diameter.

*The butter fat was prepared by melting unsalted butter at 65° C., decanting the oil portion from the curd and water, and shaking with anhydrous Na_2SO_4 while warm. The fat was then filtered through a Buchner funnel using quantitative paper. The product was entirely clear. The butter was furnished through the courtesy of the H. P. Hood and Sons Co., Boston, Mass.

TABLE II. COMPARATIVE STUDIES OF FAT EMULSIONS WITH REGARD TO TYPE OF FAT AND RATIO OF FAT TO PHOSPHATIDE EMULSIFIER

EMULSION	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
Corn oil (Gm./100 c.c.)	–	–	10	20	30	30	30	–	–	–	30	–	–	–	–	–	–	–	–	–
Coconut oil (Gm./100 c.c.)	15	10	–	–	–	–	–	30	30	30	–	30	30	15	15	15	15	15	–	–
Butter fat (Gm./100 c.c.)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	15	30
Asolectin (Gm./100 c.c.)	2.7	2.0	2.0	4.0	6.0	0.5	3.0	3.0	3.0	0.4	3.0	6.0	6.0	0.1	0.15	0.15	3.0	0.15	0.5	3.0
Na_2HPO_4 (Gm./100 c.c.)	0.6	–	0.4	0.6	1.2	–	–	–	0.6	0.6	–	–	0.33	0.002	–	–	–	–	–	–
Dextrose (Gm./100 c.c.)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3.5	–	–	–	–
Volume (c.c.) × 100	3	3	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	6	5	4
Pressure (lbs./sq. in.) × 100	20	20	20	20	20	25.5	30	30	27	20	20	20	20	20	20	20	20	20	20	20
Time (min.)	35	35	35	35	35	25	35	65	35	38	60	60	35	30	35	30	30	30	35	35
Diameter of particles (μ)	<2	<2	<3	<3	<3	<10	<2	<2	<2	<8	<2	<3	<2	<2	<2	<2	<2	<2	<4	<2
Effect of autoclaving*	N	N	N	N	N	B	N	N	N	C	N	N	L	L	N	N	N	N	N	N

*N, None; B, broke; C, creaming; L, some larger particles formed.

Of primary importance was the continuous recirculation of the material through the machine for periods of time ranging from thirty to ninety minutes, depending upon the fat content. Shorter time intervals were inadequate and also negated the advantage of using high pressures. Thus, periods of five to ten minutes yielded no better emulsions at pressures above 3,000 pounds per square inch than were obtained at 2,000 pounds per square inch. Pressures above 3,000 pounds per square inch, however, when used for longer periods of time, resulted in a shift in the mean particle size toward the lower limit of visual microscopy. Such emulsions took on a bluish appearance and transmitted red light even when the fat content was as high as 30 per cent. Light-field microscopic examination of a thin layer of an undiluted 15 or 30 per cent fat emulsion revealed relatively few discernible particles. Dark-field examination disclosed many additional particles below 0.5 μ in diameter. These characteristics indicated that most of the emulsion was a true colloidal suspension of fat in water.

The data in Table I show that high temperature was conducive to better and more rapid emulsification. When the homogenization was carried out at pressures above 3,000 pounds per square inch, the temperature rose to the boiling point provided no cooling water was used. This circumstance was helpful, for both a high pressure and a high temperature were obtained simultaneously. Experience has shown that boiling may cause the emulsion to break to a certain extent, probably due to loss of the water phase by surface evaporation. The beneficial effect of higher temperatures is believed to be due to lowered viscosity which permits a smaller valve opening at any given pressure, lowered interfacial tension, and a decrease in the force necessary to shear the fat globules.

The levels of emulsifier and fat and the ratio between them were also found to be of great importance. In general, the lower the fat level, the shorter the length of time necessary for complete emulsification and the more stable the finished emulsion to high electrolyte concentration and to autoclaving and storage. Thus, a 15 per cent coconut oil, 3 per cent phosphatide emulsion (Emulsion 31) was prepared in thirty minutes and was unaffected by autoclaving. Emulsion 22 which contained 30 per cent coconut oil and 3 per cent phosphatide required sixty-five minutes of homogenization but was stable to autoclaving. Raising the level of stabilizer to 6 per cent made little difference in the homogenizing time required to produce a good emulsion and one stable to autoclaving. Emulsions containing 0.15 per cent phosphatide were stable to autoclaving when the fat concentration was below 15 per cent. Such emulsions, however, were broken by low electrolyte concentrations.

Added dextrose has proved satisfactory as a means of rendering the emulsions compatible with blood from the standpoint of tonicity and does not adversely affect the emulsion during autoclaving. Dextrose has the added advantage of contributing to the total caloric content. Extensive animal testing has been conducted using an emulsion similar to Emulsion 22. The composition and caloric content of Emulsion 22 is given in Table III.

TABLE III. COMPOSITION AND CALORIC CONTENT OF FAT EMULSION 22

COMPONENTS	GRAMS	CALORIES
Oil	300	2700
Dextrose	35	140
Phosphatide emulsifier	30	210 (approx.)
Water	634	–
Total		3050

Studies are in progress on the chemical and physical stabilities of these emulsions and on their pyrogenic properties. Also being investigated are possible costabilizers and other emulsifiers. A serious criticism of many surface active substances studied is the tendency to in vitro hemolysis even in the low concentrations necessary. The use of filtration through bacterial filters is being studied. It has been found that 30 per cent fat emulsions can be passed through filtering candles or pads without adversely affecting stability.

The use of high-frequency sound waves as a means of emulsification has been studied by other workers.[5] The magnetostrictive principle is used extensively in the dairy industry in the production of homogenized milk. Our studies with sonation were not extensive but using this procedure we were unable to make emulsions which were as satisfactory for our purpose as emulsions made by a pressure homogenizer. This failure to produce good emulsions by sonation seemed to be related to two factors: first, the material could not be circulated adequately through the effective field of high-intensity cavitation; and second, the intensity of the mechanical activity of the vibrating membrane was so great that appreciable amounts of metal were dispersed into the solution.

SUMMARY

A photomicrographic apparatus and method have been developed to allow accurate determination of the size of fat particles in emulsions.

By means of high-pressure homogenization fat emulsions were prepared in which all particles were below 2 μ in diameter and most were beyond the resolving power of the light microscope.

Factors conducive to the preparation of such emulsions were high pressure, high temperature, continuous recirculation of the material being homogenized, and the proper fat-stabilizer ratio. Data on each of these are given.

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PARENTERAL NUTRITION

V. Studies on Soybean Phosphatides as Emulsifiers for Intravenous Fat Emulsions

Robert P. Geyer, Ph.D., George V. Mann, M.D., John Young, M.D., Thomas D. Kinney, M.D. and Fredrick J. Stare, M.D.
Boston, Mass.

THIS laboratory has carried on research in an effort to develop fat emulsions suitable for parenteral nutrition.[1-4] Previous reports have shown that fat emulsions given intravenously to dogs can be utilized as a source of energy, that dogs are able to handle large quantities of emulsified fat administered daily for long periods of time, that the fat can be given in a concentration as high as 30 per cent and at a rapid rate, and that the infused fat leaves the blood in a few hours. However, in all the animals previously studied granulomatous lesions and scarring of the lungs, liver, and spleen were noted in varying degree. Preliminary observations pointed to the phosphatide preparation used as an emulsifier as the principal agent responsible for these lesions.[4]

Since the lesions were produced by a soybean phosphatide preparation (Asolectin)* alone and since this emulsifier appeared to be the best of a large number studied, it was desirable to investigate it further. In all of the previous studies on this problem adult dogs had been used as the experimental animal. It would be advantageous to use a smaller laboratory animal for much of this work. The purpose of this paper is to report a chemical fractionation of the soybean phosphatide preparation which yields an emulsifying agent which will not give rise to the histopathology previously observed and to report observations on an improved fat emulsion given intravenously to adult rats and to puppies.

EXPERIMENTAL

The plan of the first part of this study was to utilize the rat as an experimental animal to assay various fractions of the phosphatide preparation, both alone and as part of the fat emulsion, for any lesion-producing properties. Both Sherman and Hisaw strains of the albino rat were used. The animals weighed from 150 to 250 grams and were approximately 3 months old. In most experiments a group of six rats plus controls were used. Each experimental animal was given a daily injection of the material to be tested for six successive days. The animals were injected through a tail vein while under light ether anesthesia. The volume injected varied from 1.5 to 2.5 ml. but in all cases amounted to 1 ml. per 100 Gm. of body weight. It was given in

From the Department of Nutrition, Harvard School of Public Health; Departments of Biological Chemistry and Pathology, Harvard Medical School; and Department of Pathology, Peter Bent Brigham Hospital.

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*We are indebted to Dr. Albert Scharf of Associated Concentrates Inc., Elmhurst, Long Island, N. Y., for generous supplies of this soybean phosphatide preparation.

thirty seconds or less. Six days following the last injection (unless otherwise noted) the animals were sacrificed by etherization and bleeding. Sections of all organs were fixed in 10 per cent formalin solution, embedded in paraffin, and stained with hematoxylin-eosin. Frozen sections of formalin-fixed materials were stained for fat with Sudan III when indicated.

The various fractions studied consisted of the so-called purified soybean phosphatide preparation (Asolectin) used in our previous studies and derivatives of this material obtained by fractionation procedures. These were observed for lesion-producing properties when injected alone and as a component of fat emulsions. The materials were prepared for intravenous ad-

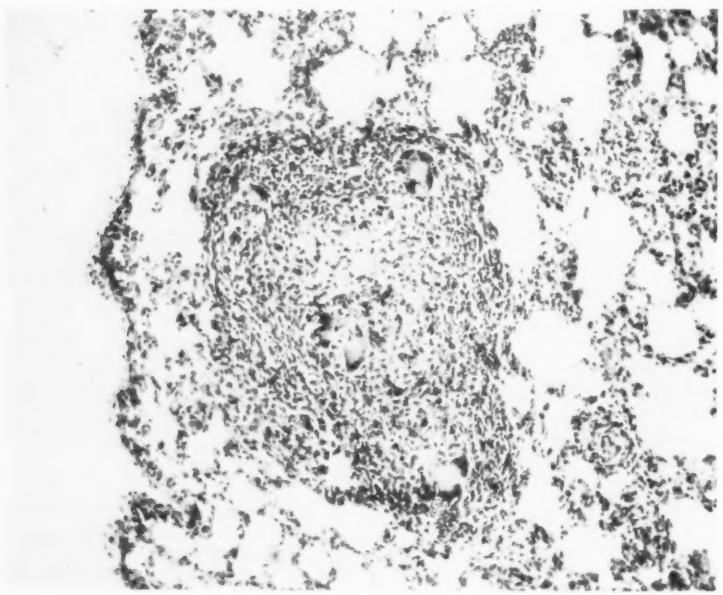

Fig. 1.—Most severe type of granulomatous lesion. Note numerous giant cells, necrosis, and polymorphonuclear leukocytic infiltration. Hematoxylin and eosin stain. ×200.

ministration using one of the following procedures. (1) Homogenized preparations were made by the method recently described for the preparation of fat emulsions.[5] The material was added to boiling water and spun for three minutes in a Waring blendor at top speed. It was then homogenized in a high-pressure homogenizer* for thirty-five minutes. The pressure was maintained at 3,000 pounds per square inch, and the temperature, at 75 to 80° C. The process was carried out under a nitrogen atmosphere. Solutions were buffered with phosphate to a pH of 7.4 and made isotonic with either glucose or sodium chloride. The material was placed in brown glass pressure bottles; the air was displaced with nitrogen; and the tightly capped bottle was autoclaved at 15 pounds per square inch for fifteen minutes. (2) Blended preparations were obtained in the same manner except that the

*Junior Viscolizer No. 50; Cherry Burrell Corporation, Chicago.

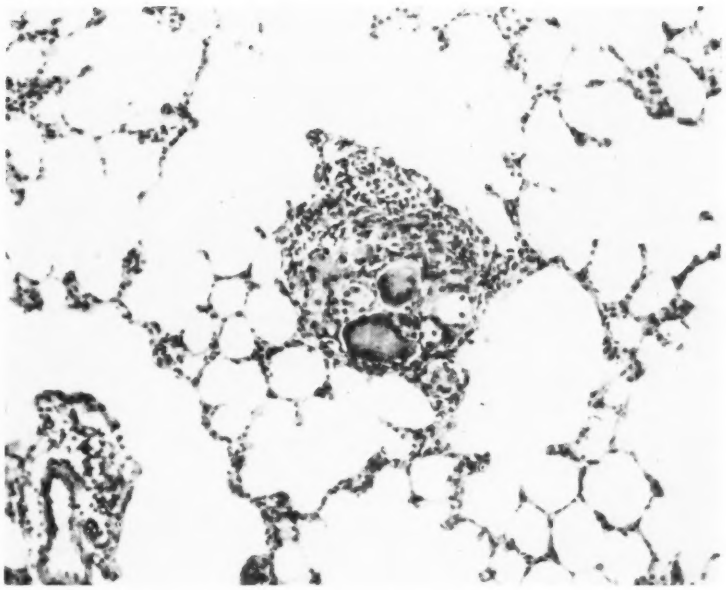

Fig. 2.—Less severe lesions with few giant cells. Note absence of necrosis and small degree of fibrosis. Hematoxylin and eosin stain. ×215.

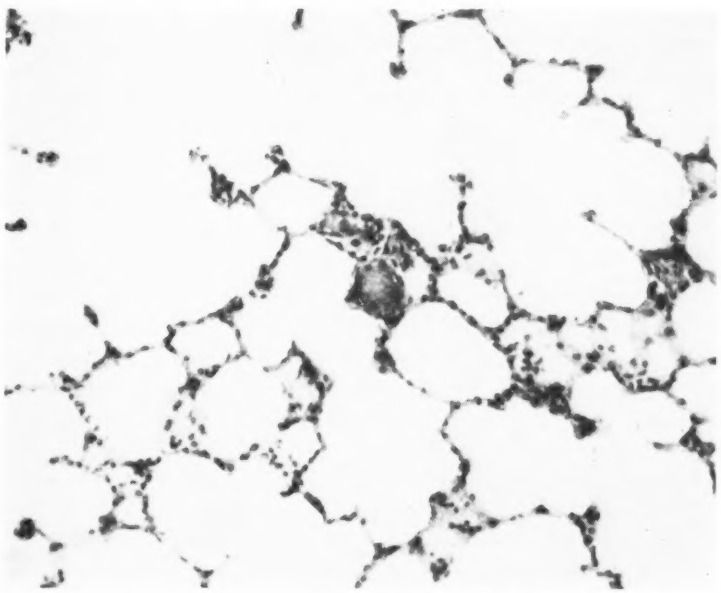

Fig. 3.—Minimal lesion made up of one to two giant cells without accompanying necrosis or fibrosis. Hematoxylin and eosin stain. ×300.

high-pressure homogenization was omitted. (3) Crudely mixed preparations were made by adding the material to water (50° C.) and spinning in the blendor at high speed for thirty seconds. Tonicity and sterility were achieved as with the homogenized preparations.

In the experiments to be described the pathologic lesions were restricted to the lung and were not evident grossly. On microscopic examination the most severe lesions were granulomatous in type (Fig. 1). The individual lesions consisted of groups of mononuclear cells and fibroblasts with one to four giant cells of the Langerhans type at the center. In the larger nodules Mallory's aniline blue stain demonstrated loose, fine, collagenous fibers arranged in concentric fashion. Seen among the fibers were large epithelioid cells. A few of the larger lesions had necrotic centers with many polymorphonuclear leucocytes. The giant cells contained from a few to twenty or thirty nuclei. These were arranged about the periphery of an abundant amount of light basophilic to eosinophilic cytoplasm. The cytoplasm was homogenous for the most part. However, some giant cells contained small droplets that stained red with Sudan III while in other giant cells particulate matter was seen. Refractile bodies were not observed when a frozen section of the tissue was examined through a polarizing microscope. Ziehl-Neelsen stain demonstrated no acid-fast material in the cytoplasm. No asteroids were seen in the cytoplasm. The nuclei had prominent nuclear membranes and centrally placed nucleoli. The lesions in the more severe cases varied in size from 0.1 to 1.0 millimeter. In some instances the nodules were confluent. They had no constant relationship to bronchi or vessels. There was steady regression in the size of the lesions in animals which were allowed to live for a longer period than that stated.

The less severe lesions were seldom over 0.5 millimeter. (Fig. 2.) There was little or no fibrosis and no necrosis. The nodules consisted of mononuclear cells and one to two giant cells, which were similar to those seen in the largest lesions.

Some of the lesions consisted of only one to two giant cells with no cellular elements or fibrosis about them. (Fig. 3.) These were seen in the alveolar walls with only a separation of the basement membranes on each side.

The sections were graded according to the severity and frequency of the giant cell lesions. Four plus lesions were the most severe. In these there were three to four nodules per low-power field which were often confluent. The lesions themselves were large, with fibrosis and some necrosis. Three plus lesions were smaller with little fibrosis and no necrosis. However, they were almost as frequently seen as the four plus, occurring from one to three times per low-power field. Two plus lesions were small and infrequent, one lesion being observed per three high-power fields. One plus lesions consisted of rare isolated giant cells with no cellular components about them.

Rat Assay Experiments, Part I.—

Experiment 1: The soybean phosphatide preparation (Asolectin) in a concentration of 3 per cent in boiling water was blended in a Waring blendor for a period of ten minutes. Microscopic examination of the material showed

that the suspended particles varied from 10 to 20 μ in size. Six adult rats were injected with this material. Sections of the lungs from all the assay animals showed numerous four plus lesions. In the center of some of the lesions there was definite necrosis and infiltration by polymorphonuclear leucocytes. These lesions were more numerous and larger than those found in any of the subsequent groups. These results and those of subsequent experiments are given in Table I.

TABLE I. SUMMARY OF RAT ASSAY EXPERIMENTS ON INTRAVENOUS ADMINISTRATION OF PHOSPHATIDE PREPARATIONS AND FAT EMULSIONS

EXPERIMENT	MATERIAL GIVEN	CONCENTRATION (%)	MAXIMUM PARTICLE SIZE (μ)	GRADING OF LESIONS RAT NUMBER 1	2	3	4	5	6
1	Blended commercial phosphatides	3	20	++++	+++	+++	++++	++++	++++
2	Homogenized commercial phosphatides	3	2	+	+	+	+	+	–
3	Homogenized commercial phosphatides	9	2	+	+	–			
4	Fraction A(F1)	3	3	+	+	+			
5	Fraction B(F1)	3	2	–	–	–			
6	Fraction C(F1)	3	5	+++	+++	+++			
7	Fraction A(F2)	0.09	5	+	+	–*			
8	Fraction B(F2)	3	2	–	–	–	–	–	–
9	Fraction B(F2)	3	5	–	+	+	-		
10	Fraction A(F2)	0.09	14	+++	+++	+++			
	Fraction B(F2)	3							
	Fraction C(F2)	1.5	9	+	+	–			
	Fraction B(F2)	1.5							
11	Aspergillus niger	--	7	+++	+++	+++	+++	+++	+++
12	Emulsion of cocoanut oil & fraction B(F2)	30 3	2	–	–	–	–	–	+
Control group (12 animals)		–	–	–	–	–	–	–	–

*Animal died one hour following last injection.

Experiment 2: The soybean phosphatide preparation (Asolectin) in a concentration of 3 per cent in water was homogenized. The particle size in this emulsion was 2μ or smaller. A group of six rats were injected. Sections of the lungs from all animals showed small, one plus lesions. There was little fibrosis or infiltration and no necrosis.

Experiment 3: The soybean phosphatide (Asolectin) in a concentration of 9 per cent in water was homogenized. It was injected into three rats daily for only two days. The animals were sacrificed on the twelfth day of the experiment. Sections of the lungs were graded one plus and were similar to the lungs of the animals in Experiment 2. This experiment was carried out to determine if the total amount of phosphatide homogenate could be given in a shorter period of time without changing the number or severity of the lesions. It was concluded that, within limits, the rate of administration of the homogenized phosphatide was not a significant factor in the production of the lesions.

Fractionation of the Phosphatide Preparation, Asolectin.—While the homogenized phosphatide preparations produced relatively few lesions it was felt that further experiments should be carried out to eliminate, if possible, the factor or factors responsible for the few lesions that were present without destroying any of the emulsifying properties of the phosphatides. Consequently, several chemical fractionation procedures were done on the purified soybean phosphatide and many of these preparations were then assayed for lesion-producing properties as in the previous experiments.

Fractionation 1 (F1): One hundred grams of soybean phosphatides were dissolved in 500 c.c. of chloroform. To this solution 1,500 c.c. of acetone were added slowly, with stirring, to yield a finely divided precipitate of phosphatides. After filtration the precipitate was redissolved in 100 c.c. chloroform and reprecipitated with 300 c.c. of acetone. After filtration the solid product was washed twice with acetone and was then freed of solvent by means of vacuum at room temperature. The combined filtrates from the preceding operations were taken to dryness under vacuum in a stream of nitrogen and yielded 18.2 Gm. of a dark, gummy material which was given the designation A(F1). The acetone precipitate was added to 350 c.c. of absolute alcohol (63° C.) and was spun in a Waring blendor at high speed for three minutes. The suspension was cooled to room temperature and placed in the refrigerator overnight (7° C.). After filtration through a Buchner funnel, the precipitate was washed with three 30 c.c. portions of cold absolute alcohol. The entire alcohol treatment was repeated except that the material was filtered after seven hours. Removal of the residual solvent from the precipitate yielded 41 Gm. of a granular, light-tan material which was designated B(F1). The combined filtrates from the alcohol treatment were concentrated in vacuum under nitrogen and furnished 19.4 Gm. of a light-yellow, amorphous substance. This was designated C(F1). The yields of both C(F1) and B(F1) were low because of loss during the initial step of this method.

Fractionation 2 (F2): It was found that a diethyl ether solution of soybean phosphatide remained turbid. This insoluble material would have appeared in fraction B(F1) of the previous procedure. Therefore, 50 Gm. of phosphatides were dissolved in 100 c.c. of diethyl ether and the insoluble particulate material was removed by centrifugation. This material was washed five times with 10 c.c. portions of diethyl ether and was then freed of solvent under vacuum. This yielded 31 mg. of a white powder which was designated A(F2). Microscopically this material comprised some particles which closely resembled rod-shaped bacteria and other particles which were of many irregular shapes and which ranged in size from less than 1 micron to 15 micra. Subsequent chemical determinations demonstrated that much of this material gives color tests characteristic of denatured proteins.

The filtrate from the preceding paragraph was made up to 200 c.c. with diethyl ether and was placed in the refrigerator (–7° C.) for forty-eight hours. A slight cloudiness developed but disappeared on warming. Fifty cubic centimeters of acetone were added and after thorough shaking the solution was

placed at −7° C. overnight. The top layer was decanted and the bottom layer was carried through the diethyl ether-acetone procedure again. After decantation, the top layers were combined and were concentrated under vacuum in a stream of nitrogen. The yield was 5.4 Gm. of a very dark, viscous, gummy substance. This comprised preparation C(F2).

The bottom layer from the previous procedure was filtered through a Seitz filter using positive pressure. Concentration under vacuum yielded 32 Gm. of a light-yellow, granular material B(F2).

Rat Assay Experiments, Part II.—

Experiment 4: A 3 per cent suspension of fraction A(F1) was prepared by mixing in a Waring blendor for three minutes with boiling water. After autoclaving this material it was injected into six adult rats daily for a period of six days. The dosage was as previously mentioned for all of these experiments. The rats were sacrificed on the twelfth day of the experiment. Sections of the lungs showed one plus lesions.

Experiment 5: A blended 3 per cent suspension of fraction C(F1) in water was injected into three rats daily for six days. The rats were sacrificed on the twelfth day of the experiment. Sections of the lungs were graded three plus for lesions. These lesions while as numerous as those found in the animals injected with blended whole phosphatide (Experiment 1) were not so large. Also, there was less inflammatory reaction and fibrosis, and foreign body giant cells were less numerous per lesion.

Experiment 7: A 9.5 mg. per cent suspension of fraction A(F2) in water was injected into three rats daily for six days. This material was prepared for injection by grinding in a mortar with 5 Gm. of dextrose and then was taken into water. The rats were sacrificed on the twelfth day of the experiment. Sections of the lungs were graded one plus for lesions.

Experiment 8: Two groups of animals were used to test a blended preparation of fraction B(F2) in which the particle size varied from 1 to 2 micra. It was injected into six rats daily for six days. These rats were sacrificed on the twelfth experimental day. Sections of the lungs showed no lesions.

Experiment 9: In the second assay with fraction B(F2) it was again made up in 3 per cent concentration in water but was crudely mixed in the Waring blendor. The particle size varied from 1 micron to 8 micra. This material was injected into four rats daily for six days. The animals were sacrificed on the twelfth experimental day. No lesions were found in the lungs of two rats. The remaining two rats had lesions graded one plus. These lesions were small with no fibrosis or cellular infiltrates.

Experiment 10: Fraction C(F2) was not suitable for injection by itself because of its high content of fatty acids and neutral fats and consequent insolubility in water. For this reason blended preparations were made up as follows: the first contained fractions A(F2) plus B(F2), and the second, fractions C(F2) plus B(F2), both fractions in a concentration of 1.5 per cent in water. The B(F2) preparation was thus used as an emulsifier for the insoluble

A(F2) and C(F2), and the total concentration of each combined fraction was 3 per cent. Three rats were given six daily injections of fractions C(F2) plus B(F2) and were sacrificed on the twelfth experimental day. The lungs were graded one plus. Three additional rats were given six daily injections of A(F2) and B(F2) and were killed on the twelfth experiment day. The lungs were graded two plus in one rat and three plus in the other two. There were a few medium sized lesions but the majority were small.

Experiment 11: It was felt that microorganisms growing in the crude products might be partially responsible for the production of lesions even though they were killed by autoclaving. The coconut oil, crude phosphatide, and water from the homogenizer were cultured. *Staphylococcus albus* and *Aspergillus niger* were recovered from the crude phosphatide. The fungus was grown in pure culture, suspended in isotonic saline autoclaved at 15 pounds pressure for fifteen minutes, and injected into six rats in a manner similar to that used in the other experiments. The rats were sacrificed on the twelfth day of the experiment. The lungs were graded three plus for lesions. Spores and hyphae were demonstrated in the giant cells. In this instance, lesions were also found in the spleen and liver.

Experiment 12: An emulsion (Emulsion 35)[5] containing coconut oil, 300 Gm., phosphatide fraction B(F2), 30 Gm., dextrose, 35 Gm., and water, 634 ml., was prepared and injected daily for six days into a group of six rats. The particle size of this emulsion was less than 2 μ, predominantly less than 0.7 micron. The dosage was 0.5 c.c. per 100 Gm. of body weight. The animals were sacrificed on the twelfth experimental day. Only one small lesion was found in one lung of the six rats examined.

Control Animals for Rat Assay Experiments: A group of twelve rats served as controls. These animals were kept in the same cage as the experimental animals and were distributed throughout the twelve experiments. The lungs were examined and no lesions such as those described were present in any of the twelve control animals. An occasional rat in both the experimental and control groups showed pneumonitis such as is commonly encountered in laboratory rats. The lesions described herein were sufficiently characteristic to be distinguished from this type of pneumonitis.

Puppy Experiments.—

Experiment 13: A more extensive study of Emulsion 35 as prepared by the improved procedure described in the previous report[5] and using fraction B(F2) prepared from soybean phosphatides was carried out in puppies. Assay in rats had indicated that this phosphatide fraction would not lead to visceral lesions when infused as a water emulsion alone in a 3 per cent concentration or when used in that concentration as a component of a 30 per cent fat emulsion.

A group of four Labrador puppies, all littermates raised in this laboratory, was used. The animals were in apparent good health. They were wormed and during the course of the experiment were given canine distemper serum at two-week intervals. Despite this precaution there were indications toward the end of the experiment that the animals were infected. Anorexia, fever, and leu-

cocytosis suggested that a respiratory, bacterial infection was present. The animals were divided into two groups. The three animals in the first group were infused daily with amounts of emulsion varying from 1.5 to 10 Gm. per kilo of body weight. The animals were fed a purified diet at the outset and were later transferred to a natural diet.* The fourth animal served as a control and was not infused.

Immediate reactions to the intravenous infusion of the emulsion were for the most part determined by the rate of the infusion. This aspect of the work and detailed metabolic studies will be reported in a later paper. Pertinent to this discussion, however, was the observation that on several occasions various dogs became dyspneic at the start of the infusion. Expiration became difficult, the animal wheezed audibly, and there were forced attempts to clear material from the trachea. In each instance auscultation revealed profuse expiratory rhonchi and wheezes. Upon discontinuation of the infusion the animal appeared well within fifteen minutes and the abnormal signs had disappeared. Examination microscopically of the emulsion involved revealed loose aggregations of particles 2 μ or less in diameter which constituted a flocculus or raft-like mass measuring up to 25 μ in diameter. The nature of the conditions leading to this phenomenon is not clear. Table II presents the data concerning the number of

TABLE II. SUMMARY OF INTRAVENOUS FAT INFUSION IN PUPPIES

PUPPY	DAYS INFUSED	TOTAL EMULSION INFUSED (C.C.)	TOTAL FAT INFUSED (GM.)	TOTAL EMULSIFIER B(F2) (GM.)	DAY OF EXPERIMENT SACRIFICED
904	0	0	0	0	52
907	30	2,343	703	70.3	49
905	42	4,704	1,411	141.1	56
909	84	10,295	3,089	308.9	84*

*Animal died after prolonged massive doses of emulsion leading to anorexia.

days the animals were infused and the amounts of emulsion and total fat infused. Puppy 904, the control animal, received no infusions; Puppy 907 received 703 Gm. of fat over a thirty-day period; Puppy 905 received 1,411 Gm. of fat over a forty-two day period; and Puppy 909 received 3,089 Gm. of fat over an eighty-four day period. All of these puppies were sacrificed for post-mortem examination at varying times as indicated in Table II. Gross examination at autopsy revealed normal appearing organs in all puppies. Sections were made of lung, liver, spleen, kidney, brain, intestine, bone marrow, adrenal and lymph nodes and were stained with hematoxylin and eosin.

Two animals (Puppies 905 and 907) were sacrificed by etherization and bleeding. Both animals had pulmonary findings consistent with a mild distemper. The lungs from both animals also showed occasional giant cell lesions which were graded one plus. No granulomatous lesions such as were described previously were found in any of the other organs. No hemosiderin deposition was noted in the spleen, liver, lymph nodes, or bone marrow. The liver was stained with Sudan III and no fat was found. There was hyperplasia of the white cell series in the bone marrow.

*Gaines Dog Meal, furnished through the courtesy of the Research and Development Department, General Foods Corporation, Hoboken, N. J.

Puppy 909 died during the night and all organs were markedly autolyzed. As far as could be determined there were no granulomatous lesions present. There was pulmonary edema and many of the smaller bronchi were plugged with mucous.

The control animal (Puppy 904) also showed pulmonary lesions consistent with mild distemper. No other lesions of consequence were present in any of the organs.

DISCUSSION

In previous studies[2-4] it was shown that dogs receiving emulsions of corn oil or coconut oil in concentrations of 15 per cent, 30 per cent, or 2 per cent developed granulomatous lesions which were most marked in the lungs and more rarely found in the spleen, kidney, and liver. Essentially the same lesions were found when the emulsifying agent, a soybean phosphatide preparation, was given intravenously without added fat.[4] In the present report it is shown that such lesions were also produced in the lungs of the albino rat under similar conditions. The liver, spleen, kidney, heart, and brain of the rats were studied but no lesions were found. The rat proved a good experimental animal for assay of the emulsions and various phosphatide fractions because only small amounts of material were required for the development of the lesions. Thus only 30 mg. of the original phosphatides per 100 Gm. of rat per day for a six-day period were required to produce numerous lesions. That the assay time could be reduced further is shown in Experiment 2 in which 180 mg. per 100 Gm. of rat given for two days also produced typical lesions. Histologic examination of the lungs seemed to provide an accurate index of the lesion-producing properties of the test materials.

The larger lesions produced in the lung were made up of mononuclear cells, fibroblasts, and foreign body giant cells. Various substances were demonstrated in some of the giant cells. Some contained neutral fat, while in others particulate matter was observed. The lesions of the rats receiving the mold showed spores and hyphae in the giant cells. In other instances small bits of leather fragments were demonstrated. The latter probably came from the leather gaskets used in the homogenizer. Preliminary experiments have suggested that by using plastic gaskets and passing the finished emulsion through a suitable filter, particulate fragments can be completely removed.

The soybean phosphatide preparation (Asolectin) used in our previous studies was fractionated to yield an emulsifying agent more suitable for intravenous use. The first fractionation of the phosphatides was unsatisfactory because the small amount of ether-insoluble material present originally was not removed prior to subsequent steps. In the second fractionation this material, fraction A(F2), was removed at the start of the procedure and consisted of particles of solid material whose greatest dimension was as large as 12 micra. Such particles would be sufficiently large to block the alveolar capillaries in addition to setting up giant cell reactions. Significantly, this fraction gave color tests characteristic of denatured proteins. Since prolonged high-pressure homogenization was fairly effective in preventing lesion formation, it

is probable that the particulate material was rendered small enough to escape being caught in the capillaries. This precludes the possibility of a chemical inactivation of the causative factor(s). The material which comprised fraction A(F2) would be present in fraction B(F1) of the first fractionation. The latter fraction caused extensive lesions. The material present in fraction C(F2) would consist of fat, fatty acids, sterols, lipid degradation products, waxes, and other substances soluble in an acetone-ether mixture. From the standpoint of lesion production this fraction was of low potency. Its removal from the phosphatides is warranted, if for no other reason than its lack of emulsifying properties.

Fraction B(F2) should consist mainly of phospholipids and represents most of those originally present, for in practice an over-all yield of 90 per cent is obtained. This fraction proved to be the most efficient for our purposes. In Experiment 8 a blended preparation with particle size varying from 1 to 2 μ was injected into six rats without producing lesions; in Experiment 12 it was injected as part of a 30 per cent fat emulsion (Emulsion 35) into six rats of which five were found free of lesions and the remaining animal showed only one small lesion in one lung. That this fraction is a good emulsifying agent is indicated by the fact that a 30 per cent coconut oil emulsion can be stabilized with 3 per cent of the fraction. Preparations of fraction B(F2) which have been kept at -7° C. for three months still retain their emulsifying properties. From Experiment 9 it can be seen that the injection of a crude mixture of fraction B(F2) in which the particles were 1 to 8 μ gave only a few one plus lesions. This suggests that within limits the particle size of these phospholipids is not of itself of great importance.

The twelve control rats for these assay experiments did not show the granulomatous lesions of the lung that had followed the injection of fractions other than B(F2).

The puppy experiments were of interest because they showed that fat could be given intravenously to this species without producing the extensive granulomatous lesions that had characterized all of our previous work. Also, these studies were our first experiments with the administration of fat intravenously to growing animals. The fat emulsion used (Emulsion 35) contained 30 per cent coconut oil and was stabilized with a concentration of 3 per cent of fraction B(F2). The fat emulsion was well tolerated by the three puppies that received it depending largely on how rapidly it was given, particularly during the first few days of infusion. The growth and metabolic data of these puppies will form part of the substance of a subsequent report.

SUMMARY

A procedure to assay fat emulsions for intravenous use and the constituents of such emulsions has been developed using the albino rat.

Using this assay procedure, confirmatory evidence was obtained that the soybean phosphatide material used as an emulsifying agent in our previous emulsions was the principal cause of the granulomatous lesions observed following the intravenous administration of fat emulsions.

Chemical fractionation of the soybean phosphatide preparation was accomplished so as to yield a fraction with good emulsifying properties and little or no lesion-producing properties.

A 30 per cent fat emulsion (Emulsion 35) was given in daily intravenous infusions to three puppies in varying amounts and for periods of time varying from thirty to eighty-four days without producing the granulomatous lesions found in previous experiments with adult dogs.

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PARENTERAL NUTRITION

VI. Fat Emulsions for Intravenous Nutrition: The Turbidimetric Determination of Infused Fat in Blood After Intravenous Administration of Fat Emulsions

Robert P. Geyer, Ph.D., George V. Mann, M.D., and Fredrick J. Stare, M.D.
Boston, Mass.

ONE phase of the research on the intravenous use of fat emulsions has been the determination of the rate of disappearance of the infused fat from the blood.[1] The usual methods for determining blood lipids are laborious and time consuming, and even the microadaptations of these methods require quantities of blood large enough to prohibit repeated use on small laboratory animals. Therefore the turbidimetric method described in this paper was developed. It has the advantage of requiring only 20 c.mm. of blood for a rapid, accurate determination. Thus, numerous samples may be taken and the animals' ability to remove infused fat from the blood stream can be easily ascertained. The term "fat tolerance curve" is proposed for the graphic representation of the data obtained.

The present paper gives the method used, proof of its validity, and the fat tolerance curves of the rat, dog, and rabbit when given 30 per cent fat emulsions.

EXPERIMENTAL

Principle of the Method.—After the intravenous infusion of a fat emulsion the blood plasma becomes turbid. This turbidity is measured in a photoelectric colorimeter* and the amount of infused fat is calculated from a standard curve or K value. Within limits the turbidity bears a straight-line relationship with the instrument readings and fat concentration. By taking successive samples of blood after an infusion of fat, a fat tolerance curve can easily be obtained by plotting the turbidity readings against time.

Materials Used.—

1. Thirty per cent coconut oil emulsion. The composition, preparation, sterilization, and determination of particle size of this emulsion (Emulsion 35) have been described previously.[2]
2. Five per cent dextrose solution.
3. Concentrated ammonium hydroxide.
4. Superoxol. Thirty per cent solution of hydrogen peroxide.

Preliminary Studies.—Originally the method used was to read the turbidity of a diluted sample of plasma in the photoelectric colorimeter using filter

From the Department of Nutrition, Harvard School of Public Health, and the Department of Biological Chemistry, Harvard Medical School.

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*A Klett-Summerson photoelectric colorimeter was used in these studies.

No. 66. This filter was chosen because the presence of small amounts of hemoglobin had little effect upon the readings. Investigation demonstrated that the turbidity read, however, was the summation of that of the fat and of extraneous turbidity caused by a reaction between the emulsion and plasma. This interfering turbidity could be removed by the addition of a small amount of ammonium hydroxide without influencing the fat emulsion. It was also found that the emulsion component responsible for the false turbidity reading lay in the phosphatide preparation used. Microscopic dark-field examination of a mixture of phosphatide and plasma revealed many nonspherical, highly refractile bodies which could be made to dissolve by touching the edge of the liquid under the cover glass with ammonium hydroxide. This phenomenon was not observed with either the phosphatide or the plasma alone. The following experiment was carried out to study quantitatively the reaction between the plasma and the phosphatide and also to determine whether the ammonium hydroxide influenced the fat emulsion. A 30 per cent fat emulsion was diluted with water and 4 ml. samples were pipetted into standard Klett tubes. Various amounts of clear dog plasma were added and after thorough mixing the tubes were filled to the 5 ml. mark with water. The turbidities were read and then to each tube was added 0.05 ml. of concentrated ammonium hydroxide. After mixing, the turbidities were again read. The results of this experiment are shown in Fig. 1.

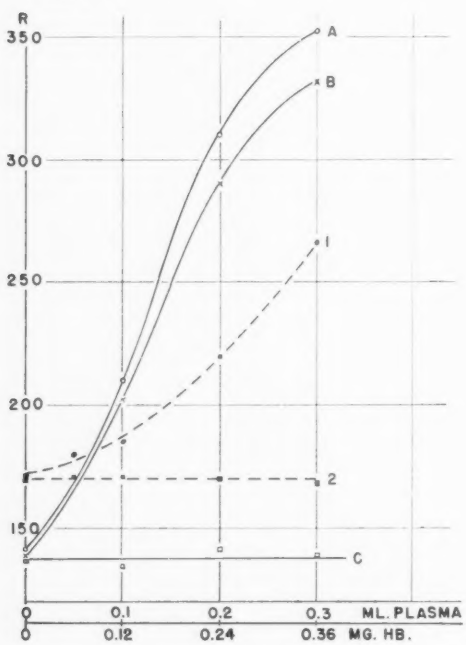

Fig. 1.

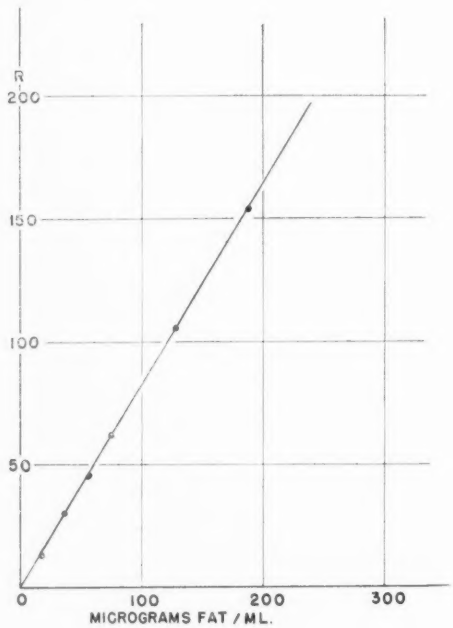

Fig. 2.

Fig. 1.—The effects of plasma and hemoglobin on the turbidity of fat emulsions. *1*, Plasma plus emulsion; *2*, plasma plus emulsion after NH_4OH treatment; *A*, hemoglobin plus emulsion; *B*, hemoglobin plus emulsion after NH_4OH treatment; *C*, hemoglobin plus emulsion after NH_4OH and H_2O_2 treatment; *R*, instrument reading.

Fig. 2.—Standard curve showing instrument readings (*R*) plotted against fat concentration in diluted emulsion. (Above *R*, or 220, the curve is no longer linear.)

In an effort to make the method more sensitive without necessitating the use of larger blood samples, two changes were made. First, the standard Klett tubes were replaced with microtubes, and second, filter No. 42 was used in place of No. 66. To remove any hemoglobin which might be present various oxidative reagents were tried and it was found that hydrogen peroxide was most satisfactory. The following experiment illustrates the effect of the peroxide treatment on the hemoglobin and the fat emulsion. Four milliliter aliquots of a diluted emulsion were placed in standard Klett tubes to which were added various quantities of a hemoglobin solution containing 2.9 mg. of hemoglobin per milliliter. The tubes were brought to the 5 ml. mark with water and the turbidities were read using filter No. 42. To each tube was added 0.05 ml. of Superoxol and the tubes were heated in a water bath at 60° C. for five minutes. After cooling to room temperature the turbidities again were read in the colorimeter. The results are shown in Fig. 1. It is necessary that the tubes be cooled to room temperature before turbidity readings are taken; otherwise the results will be significantly low.

Adopted Procedure.—By means of a hemoglobin pipette, 20 c.mm. of blood are collected and discharged into 2 ml. of 5 per cent dextrose solution contained in a 13 by 100 mm. Pyrex test tube and the pipette is rinsed in the usual manner. If any clotting occurs the sample should be discarded. The contents of the tube are well mixed by rotation and the tube is centrifuged at 1,300 revolutions per minute for ten minutes. The supernatant is decanted into a Klett microtube, care being taken to avoid carry-over of the sedimented cells, and 0.05 ml. of concentrated ammonium hydroxide is added; after mixing, 0.05 ml. of Superoxol is added. The tubes are heated in a water bath at 60 to 65° C. for four minutes and then cooled to room temperature. Any moisture which has condensed on the sides of the tube is mixed in, and any bubbles of gas attached to the sides of the tube are removed by gentle tapping. The turbidity is read in the colorimeter using filter No. 42.

A standard curve is obtained by making various dilutions of the emulsion using 5 per cent dextrose solution as the diluent. Two milliliter quantities of each dilution are placed in the microtubes and the tubes are carried through the procedure outlined in the preceding paragraph. The turbidities obtained are plotted against the concentration of fat. Where greater accuracy is required the standard curve can be made with 1.5 ml. samples, and in the procedure for the unknown, instead of simply decanting as much of the supernatant as possible, a 1.5 ml. aliquot can be taken. This would make the dilution effect of the reagents a constant throughout. In routine practice, however, it is sufficient to use the method outlined. A typical standard curve is given in Fig. 2.

Comparison With Microoxidation Method.—A rabbit weighing 4.3 kilograms was given an injection by ear vein of 12 ml. of the emulsion in the course of two minutes. At intervals, 2 ml. samples of aural venous blood were taken and immediately heparinized. After centrifuging at 1,300 revolutions per minute for fifteen minutes, 20 c.mm. of the plasma were added to 2 ml. of 2 per cent dextrose solution contained in Klett microtubes. The tubes weer carried

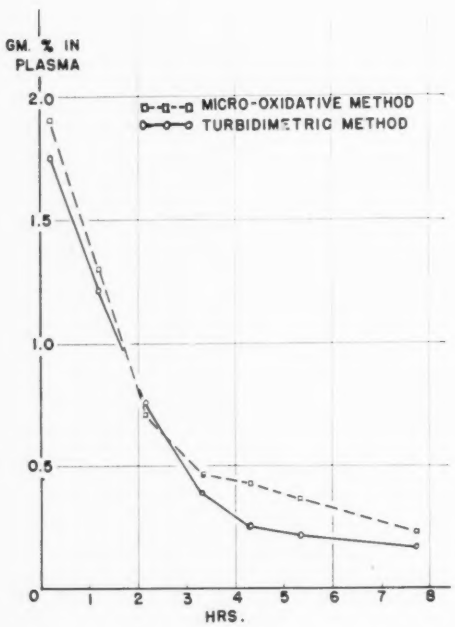

Fig. 3.

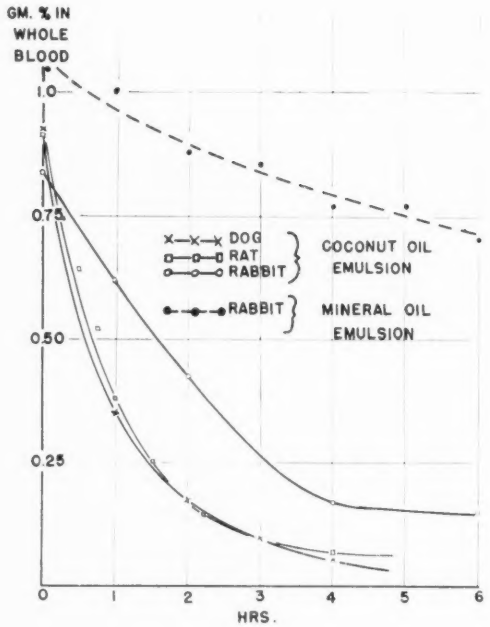

Fig. 4.

Fig. 3.—Comparison of microoxidation method and turbidimetric method of plasma fat following intravenous administration of fat emulsion.

Fig. 4.—Fat tolerance curves of various species following intravenous administration of 1½ Gm. of fat per kilo of body weight.

through the regular procedure and the turbidities were read in the colorimeter. Concurrently, suitable aliquots of each of the plasma samples were taken and carried through Boyd's modification[3] of Bloor's microoxidation method for total fatty acids plus cholesterol. The results of this experiment are given in Fig. 3.

Fat Tolerance Curves.—The tolerance curves of three species of animals to a 30 per cent coconut oil emulsion (Emulsion 35) and of one species (the rabbit) to a 30 per cent mineral oil emulsion were determined. Several rabbits, dogs, and rats each were given 5 ml. of emulsion per kilogram of body weight at the rate of 2 ml. per minute. The rabbits were injected by ear vein, the dogs by leg vein, and the rats by tail vein. Turbidity curves were obtained on venous blood using the procedure given previously. The results of these experiments are shown in Fig. 4 where each curve represents one animal of each species.

DISCUSSION

The turbidimetric determination of infused fat in the blood following intravenous administration of a fat emulsion can be quickly accomplished with only 20 c.mm. of blood. It is essential that the emulsion be stable in the blood and to subsequent treatment with the reagents used. It is equally important that hemoglobin and extraneous turbidity be removed. As shown in Fig. 1 and Table I these conditions have been adequately met. That the values agree well with a conventional microoxidation method is shown in Fig. 2. It is to be ex-

pected that the curve obtained by turbidimetric means would be lower than that found by the microoxidation method, especially as the latter has been employed here, since total fatty acids plus total cholesterol have been determined. The turbidimetric method determines principally the infused neutral fat, because the normal turbidity of the plasma lipids is extremely low. That a change in instrument and conditions might allow the determination of the normal colloidal lipids in plasma is indicated by the report of Moreton.[4] In several instances chylomicrographs[5] have been determined simultaneously with the turbidity curves after a fat emulsion injection. Agreement between the resulting curves was good.

The reaction between the phosphatide and plasma which gives rise to the extraneous turbidity observed may be similar to that reported by Chargaff and Ziff[6] to occur between cephalin and basic proteins. A large percentage of the phosphatide is made up of cephalins, and in vitro tests have shown that when the pH of a mixture of plasma and phosphatide is lowered, a voluminous precipitate is formed. Neither the plasma nor phosphatide alone will form such a product. The resulting precipitate when washed with water, acetone, and petroleum ether was insoluble in water but readily dissolved in a weak ammonium hydroxide solution. Reprecipitation results if the solution is acidified. What plasma protein is involved is unknown at present. In agreement with the findings of Chargaff and associates[7] is the fact that the phosphatide reacts with oxyhemoglobin and on acidification yields a reddish-brown precipitate. The latter is soluble in dilute ammonium hydroxide and this may explain why, as shown in Fig. 1, the turbidity decreased slightly when NH_4OH was added even though no plasma was present.

TABLE I. PER CENT RECOVERY OF FAT FROM MIXTURES OF PLASMA AND FAT EMULSION

TUBE	DILUTED EMULSION (ML.)	PLASMA* (ML.)	5 PER CENT DEXTROSE (ML.)	TURBIDITY READING†			RECOVERY (%)
				BEFORE	AFTER NH_4OH ADDITION	AFTER H_2O_2 ADDITION	
1	0.2	--	9.8	190	190	187	--
2	0.2	--	9.8	188	188	184	--
3	0.2	0.2	9.6	196	190	187	101
4	0.2	0.5	9.3	176	205	195	101
5	0.2	0.8	9.0	186	214	197	--
6	0.2	1.0	8.8	194	216	202	98.1
7	--	0.5	9.5	18	18	8	--
8	--	1.0	9.0	37	37	20	--

*Rabbit plasma.
†Instrument readings made with filter No. 42.

The removal of the hemoglobin by the hydrogen peroxide is complete when the amount of hemoglobin does not exceed that normally present. Where hemolysis is excessive, recourse to filter No. 66 can always be taken. It is usually possible to avoid mechanical lysis of the cells. The fact that the peroxide treatment has no effect upon the emulsion is further indication of the latter's stability.

The term fat tolerance curve designates the rate at which infused fat leaves the blood stream, and it is possible that like the glucose tolerance curve it may

become of diagnostic importance. Studies are now in progress on this phase of the problem. Such curves are a convenient guide in the use of fat emulsions for intravenous nutrition. It is of interest that the rabbit, which normally ingests little fat, cleared the infused fat at a slower rate than either the rat or dog whose diet is usually higher in fat (see Fig. 4). The slow removal of the mineral oil emulsion is also of interest because the oil concerned is non-hydrolyzable. Further work with this oil may reveal the reason for this slow removal. Unlike the blood lipid curve which results after oral fat intake, the curve obtained after a fat emulsion infusion is not subject to the influence of new lipid continually entering the blood. Perhaps by knowing the animals' ability to use infused fat, the curve obtained by oral fat ingestion could be corrected to give a clearer picture of the rate of absorption from the intestine. This would assume that normal blood fat and infused fat are handled by the body in the same manner.

SUMMARY

A simple turbidimetric method is described for determining the level of infused fat in the blood after intravenous administration of a fat emulsion.

The effects of hemoglobin and extraneous turbidity are discussed and the method of their removal is given.

The method is compared with the microoxidation method for blood lipids.

Fat tolerance curves are given for the dog, the rat, and the rabbit. The difference between the behavior of mineral oil and coconut oil in the rabbit is shown.

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STUDIES ON THE CONGLUTINATION TEST IN ERYTHROBLASTOSIS FETALIS

ALEXANDER S. WIENER, M.D., AND EVE B. GORDON
BROOKLYN, N. Y.

THE original method[1] (blocking test) of demonstrating univalent antibodies (blocking antibodies or glutinins) was indirect and therefore relatively insensitive. The conglutination test[2, 3] for univalent antibodies, on the other hand, is a direct and more sensitive test and therefore has proved more satisfactory. While in saline media univalent Rh antibodies merely "coat" Rh-positive cells by combining with the specific antigen without producing any visible reaction, in plasma or serum media clumping of the coated red blood cells occurs. As has been demonstrated in previous papers,[2-5] this clumping is not due to agglutination but to conglutination. The plasma contains a third component, conglutinin, a colloidal complex of plasma proteins, which is adsorbed by the specifically sensitized red blood cells, causing them to stick together. On the other hand, agglutination in saline media is as distinct as or more distinct than in plasma media because the agglutinins (bivalent antibodies) link the cells together directly without the intervention of any third component.

On the basis of these considerations, it was postulated that univalent antibodies are composed of smaller molecules than bivalent antibodies and therefore traverse the placenta more readily than the latter.[2, 4, 6] Ample direct and indirect evidence[7, 8] confirming this prediction has now been obtained, showing that univalent Rh antibodies as well as univalent alpha and beta and other antibodies pass through the placenta early in the third trimester of pregnancy and accumulate in the fetal body until the titers in the fetal and maternal plasmas become equal. Where the fetus is Rh positive or belongs to an incompatible A-B group, the antibodies are first taken up by the red blood cells or tissues and only after these are coated do free antibodies accumulate in the plasma. In these cases, if the baby is born alive, the red blood cells at birth are coated with univalent antibodies, and it was found that this could be demonstrated simply by the conglutination technique.[2, 15] One of the purposes of this paper is to describe the results obtained with this test in a series of cases of Rh sensitization, and to compare the test with the antiglobulin method of Coombs and co-workers[9] and Hill and Haberman.[10]

At this point it may be of interest to mention other predictions made on the basis of the conglutination theory[2, 3] which have been confirmed subsequently. A characteristic of erythroblastosis fetalis is that many babies appear normal or only mildly affected at birth, but within a few hours jaundice becomes evident and rapidly increases in intensity, and the disease often terminates with the death of the infant within a day or two; such infants usu-

From the Transfusion Division of the Jewish Hospital of Brooklyn and the Serological Laboratory of the Office of the Chief Medical Examiner of New York City.
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ally present the postmortem findings of kernicterus and liver necrosis. This sequence of events occurs whether delivery takes place at term or whether the pregnancy is terminated prematurely by cesarean section or by induced labor. This indicates that the birth itself must precipitate the progress of the disease. It was reasoned that while the child was in utero the fetal cells did not clump because fetal plasma must be deficient or lacking in conglutinin. However, the profound physiologic changes occurring at birth could result in abrupt increase in the conglutinin content of the plasma causing the coated red blood cells to clump (by conglutination), thus blocking the circulation to vital organs. Direct measurement[5] of the conglutinin content of fetal plasma confirmed this prediction that the amount present is small and that the conglutinin content increases after birth, though not to the level characteristic for adult plasma. It is interesting that complement has a similar development, considering that complement has an analogous role in serologic lysis to that of conglutinin in the conglutination reaction.

Still another surmise made in connection with the conglutination theory is that conglutinin is probably identical with or related to the x-protein of Pedersen.[11] According to Pedersen, x-protein is a reversibly dissociable complex of albumin and globulin, present only in concentrated plasma or serum because slight dilution with aqueous solutions causes it to dissociate into its constituent molecules of albumin and globulin. In parallel with the behavior of x-protein, we found that relatively slight dilution of plasma with isotonic aqueous solutions causes it to lose its conglutinating activity. Conversely, we reasoned that it should be possible to produce conglutinin by mixing solutions of albumin and globulin. We did find[5] that when a 4.6 per cent solution of human globulin and a 12.5 per cent solution of human albumin, both of which had little or no conglutinating activity, were mixed so as to produce a solution with a total protein content and albumin-globulin ratio equivalent to that of normal blood serum, the resulting mixtures proved to have a high conglutinating activity, even exceeding that of normal plasma. Here again the analogy between conglutinin and complement holds, since this may be compared with the experiments with so-called midpiece and endpiece.

Obviously the sensitivity of the conglutination test will depend not only on the titer of the univalent antibody but also on the quality of the conglutinin. While the original description of the test called for the use of inactivated serum, we soon adopted the use of oxalated plasma[4] because the latter has a higher conglutinating activity and is relatively free of rouleau-forming properties. Moreover, it was found that while pure albumin itself was inferior to oxalated plasma, nevertheless the addition of small amounts of albumin to plasma enhanced the latter's conglutinating activity,[5] presumably because the added albumin combined with the natural conglutinin in the plasma to form a more active complex. Based on this observation, the albumin-plasma conglutination test was devised,[3] which must not be confused with the albumin test of Diamond and Denton[12] since the latter calls for a pure 25 per cent solution of human albumin or a pure 30 per cent solution of bovine albumin without any plasma.

MATERIALS AND METHOD

The material which formed the basis for the present study consisted mainly of infants born to Rh-negative women who had been studied during the prenatal period for the presence of Rh sensitization. Our series is a selected one in that many of the pregnant women were referred to us either because they had previously had erythroblastotic infants or because they had been found to be Rh negative in the course of routine Rh antibody examinations. In a few cases we were not called to see the affected infants until after birth, either because antenatal tests had not been done or because the mother had been incorrectly typed during pregnancy as Rh positive.

At the first interview, the expectant mother's blood was completely classified for blood group, M-N type, and Rh-Hr type, and the same tests were made on the husband to determine, if possible, whether he was homozygous of heterozygous for the Rh factor. Moreover, antibody tests were done periodically on the prospective mother's serum, and, based on the Rh-Hr tests and antibody titrations, predictions were ventured as to the type and severity of

the disease in the infant. The affected infants were mostly treated by exchange transfusion, as will be reported elsewhere.[13] As also will be shown in a separate report, the manifestations closely corresponded to the predictions and in a number of cases the infants were delivered prematurely by cesarean section or by induced labor in order to prevent stillbirths and to treat the infants by exchange transfusions.[14] Where possible, samples of the cord blood were obtained in order to test the red blood cells for coating with univalent antibodies and to examine the infant's serum for free Rh antibodies and to determine the icterus index. In the few cases where the cord serum could not be obtained because the infant was seen after birth, these determinations were carried out on the first sample obtained at the exchange transfusion.

The Rh antibody titrations on the maternal and infant sera were carried out by the agglutination and albumin-plasma conglutination methods, as described in previous papers.[3, 4] In some cases the sera were also titrated by the plasma conglutination method and blocking method, but these titers are not included in Table I in order to avoid complicating the presentation. All titrations were done at least two times against test cells of types Rh_1 and Rh_2 and the titer values obtained were averaged. In testing for coating of the fetal cells, 2 drops of a 2 per cent saline suspension of the red blood cells were centrifuged and the supernatant fluid removed completely and replaced by a drop of compatible plasma or albumin-plasma mixture.[2, 5, 15] The red cells were resuspended and after incubating for forty-five minutes at body temperature the preparation was examined for the presence of clumping. The saline suspension itself of the infant's cells invariably showed no trace of clumping. Control tests were always carried out on normal red cell suspensions to show that the plasma-albumin mixture was incapable of clumping such cells. For the antiglobulin test, an anti-human precipitin serum was absorbed with washed, packed, and pooled A_1 and B cells to remove all heteroagglutinins. The actual tests were carried out by adding this reagent (diluted 1:2 or higher, depending on its titer) to 1 drop of thrice-washed saline suspension of infant's red blood cells, readings being taken after forty-five minutes of incubation at body temperature.

The icterus index determinations were made by the acetone precipitation method.

Titrations of sera containing univalent antibodies by the antiglobulin technique were carried out in the following manner. To a series of tubes containing 1 drop each of a series of progressively doubled dilutions of the serum, 1 drop of a 2 per cent saline suspension of Rh-positive cells was added and the mixtures allowed to react in the water bath for one hour. The red cells were then washed three times with saline solution. After the third washing the sediments were resuspended in 1 drop of saline solution, and 1 drop of the absorbed anti-human precipitin serum was added. The mixtures were incubated for another hour after which the reactions were read.

RESULTS

Nine different sera from sensitized Rh-negative women were titrated by the blocking, plasma conglutination, albumin-plasma conglutination, and antiglobulin techniques in order to compare the relative sensitivities of these methods of detecting univalent Rh antibodies (see Table I). Two of the sera tested (Sera 8 and 9) contained agglutinins as well as univalent antibodies. In accordance with our previous report,[3] the blocking test gave the least sensitive results, and the plasma conglutination test was on the average about ten to fifteen times as sensitive as the blocking test, while the albumin-plasma conglutination test was on the average about four times as sensitive as the plasma conglutination test. As shown in Table I, the antiglobulin technique gave results roughly corresponding to those obtained by the plasma conglutination test, being less sensitive than the albumin-plasma method.* It is felt that the

*These experiments were repeated several times using at least three different percipitin sera for the antiglobulin test, always with similar results.

TABLE I. COMPARISON OF THE RELATIVE SENSITIVITIES OF THE CONGLUTINATION AND ANTIGLOBULIN METHODS OF TITRATING UNIVALENT ANTIBODY

SERUM	ANTIBODY TITERS (UNITS*) BY THE METHODS OF				
	AGGLUTINATION	BLOCKING	PLASMA CONGLUTINATION	ALBUMIN-PLASMA CONGLUTINATION	ANTIGLOBULIN TECHNIQUE
1	0	15		520	20
2	0	3	23	135	47
3	0	1½	2½	45	6
4	0	0	4	44	6
5	0	0	5	36	4
6	0	1	25	28	5
7	0	½	11	17	12
8	7	0	20	62	7
9	12	0	12	28	5

*The figures given represent the average of two or more titrations.

albumin-plasma method is to be preferred to the antiglobulin technique, since the former is much simpler to perform and gives more sensitive results.

In Table II are summarized a series of cases in which Rh-negative mothers and their infants were studied serologically for evidence of Rh sensitization. For clarity in discussing the findings, the cases have been divided into four groups as follows:

1. Eleven cases in which the maternal serum contained univalent antibodies without detectable agglutinins. They are arranged according to the titer of the antibodies immediately prior to or following delivery.

2. Eight cases in which the maternal serum contained Rh agglutinins with or without Rh univalent antibodies.

3. Three cases of sensitized Rh-negative women who gave birth to normal Rh-negative infants.

4. Four control cases of nonsensitized Rh-negative women with normal Rh-positive infants.

We have omitted from this table those cases in which the mother was sensitized to the A and B factors as well as to the Rh factor; these complicated cases of double sensitization will be discussed in a later paper. Two cases (Cases 16 and 25) have been included in the table in which the baby's blood group was incompatible with the mother's, but in these cases the maternal anti-A and anti-B titers (by the conglutination as well as by the agglutination technique) were lower than average, showing that the mothers were not sensitized to the A or B factors.

It would be expected that in all cases where the maternal serum contains a high titer of univalent antibody the infant would be born with its red cells completely coated with blocking antibodies. Where the maternal antibody titer is low, on the other hand, the red cells of the erythroblastotic infant would be expected to be only partly coated. As shown in Table II, this prediction was actually fulfilled in our series of cases. Thus, the Rh-positive red cells of the erythroblastotic infants of the most strongly sensitized mothers (Cases 1, 2, 3, 4, 5, 6, 7, 10, 12) failed to clump in anti-Rh_0 agglutinating serum, due to complete blocking of the cells. Moreover, with two exceptions (Cases 1 and 3) these

TABLE II. RESULTS OF SEROLOGIC TESTS ON A SERIES OF RH-NEGATIVE WOMEN AND THEIR INFANTS

CASE	MOTHER'S BLOOD TYPE	Rh ANTIBODIES IN MATERNAL SERUM (UNITS)		BABY'S BLOOD TYPE	COATING TEST ON INFANT'S RED CELLS			TITRATION OF FREE Rh ANTIBODIES IN INFANT'S SERUM (UNITS)		ICTERUS INDEX (CORD SERUM)
		AGGLUTINATION METHOD	ALBUMIN-PLASMA CONGLUTINATION METHOD		IN PLASMA	IN ALBUMIN PLASMA	COOMBS TEST†	AGGLUTINATION METHOD	ALBUMIN-PLASMA CONGLUTINATION METHOD	
1*	A_1MNrh	0	1400	$AMRh_2$†	−	−	+ +	0	400	50
2*	A_1Mrh	0	112	A_1MRh_2†	+ ±	+ +		0	4	64
3*	A_1Nrh	0	45	$OMNRh_1rh$†	−	−	+ +	0	18	44
4‖	BMNrh	0	40	$OMNRh_1rh$†	+ +	+ +	+ +	0	1½	50
5‡‖	A_1MNrh	0	40	$OMRh_0$†	+	+ +	+ +	0	2	70‡
6‡‖	A_1Mrh	0	39	$OMNRh_1rh$†	+ ±	+ +		0	3	64‡
7‖	A_1Nrh	0	32	A_1MNRh_1rh†	+	+ ±	+ +		½	20
8‖	A_1Mrh	0	30	A_1MRh_1rh	+ +	+ +		0	4	20
9	OMNrh	0	25	$OMNRh_1rh$	+ +	+ +				60
10‖	A_1MNrh	0	16§	$AMRh_1rh$†	+	+ ±		0		35
11‖	A_1MNrh	0	1	$ONRh_1rh$	−	−		0	0	14
12*	OMNrh	42	40	$OMNRh_1rh$†	+ +	+ +		0	3	20
13‡‖	OMNrh	20	70	$OMNRh_1rh$	±					60‡
14‡‖	OMrh	25	40§	$OMNRh_1rh$	−					45‡
15‖	OMrh	22	20	$OMNRh_2$	−	−		0	0	12
16‖	BMrh	4	12	$ABMNRh_1rh$	+	+ +	±	0	1½	12
17‖	A_1Mrh	7	10	A_1MRh_1rh	−	Trace	−	0	0	24
18‖	A_1BMNrh	1	2	$BMRh_1rh$	−	+ ±				24
19‡‖	BMNrh	1	1	$ONRh_1rh$	−	−		0	0	112‡
20	A_1Nrh	0	22	A_1Nrh	−	−		0	20	10
21	A_1Mrh	0	7	OMrh	−	−	−	0	7	10
22	A_1Mrh	24	11	A_1MNrh	−	−		0	2½	10
23	OMrh	0	0	$OMRh_1rh$	−	−				6
24	BMNrh	0	0	$BMNRh_1rh$	−	−				8
25	OMrh	0	0	$AMRh_1rh$	−	−		0	0	10
26	A_1Mrh	0	0	$AMNRh_1rh$	−	−				14

*These patients died despite treatment by exchange transfusion.

†These blood samples failed to clump in anti-Rhō agglutinating serum due to blocking.

‡These patients were seen for the first time twenty-four hours after birth.

§These titers represent results of plasma conglutination tests, since the cases were seen before the albumin-plasma technique was developed.

‖These babies were treated by exchange transfusion and recovered.

cells clumped (conglutinated) when suspended in compatible plasma or in albumin-plasma mixture.

The failure of the red cells of the infants of Cases 1 and 3 to conglutinate is contrary to expectation and calls for an explanation. The following plausible hypothesis suggests itself. As has already been demonstrated,[2, 5] fetal plasma is deficient in conglutinin in comparison with adult plasma. Also, there is reason to believe that fetal conglutinin differs in quality as well as quantity from adult conglutinin. Thus one may postulate that fetal plasma contains conglutinoid, a substance analogous to so-called complementoid.[16] In cases in which the fetal red cells are strongly sensitized by univalent antibodies, they will absorb conglutinoid from the fetal plasma, but conglutinoid, unlike conglutinin, fails to clump the cells, just as complementoid is adsorbed by sensitized cells but fails to lyse them. When an infant's sensitized red cells, which have adsorbed conglutinoid, are suspended in plasma they will fail to clump or clump only feebly because the conglutinoid will block the adsorption of conglutinin. Supporting this concept is the observation that such red cells are clumped strongly by antiglobulin serum even though the antiglobulin technique is ordinarily less sensitive than the albumin-plasma conglutination technique for demonstrating univalent antibodies, as has already been pointed out (Table I).

In accordance with expectations, when the maternal univalent antibody titer is very high, not only are the infant's red cells completely blocked but also free circulating antibodies can be demonstrated in the fetal plasma. Where the maternal antibody titer is low, the infant's red cells are not blocked, but partial coating of the red cells can be demonstated by the more sensitive conglutination technique (see Cases 8 and 9, Table II).

Where the maternal serum contains bivalent Rh antibodies, one would expect that the infant's red cells at birth would not be coated with univalent Rh antibodies. As is shown in Table II, this was true in only half the cases in our series. To account for coating of the infants' cells in Cases 12, 13, 16 and 18, one must postulate that the maternal serum contained univalent as well as bivalent Rh antibodies but that the former were weaker so that they were masked by the agglutinins. In support of this idea, it may be pointed out that in two cases (Cases 13 and 16) the infants' sera contained free univalent Rh antibodies.

Of special interest are Cases 20, 21, and 22 where sensitized Rh-negative women gave birth to Rh-negative children. As expected, none of these infants were erythroblastotic. In two of the cases where the maternal serum contained pure univalent antibodies, the infants' sera contained univalent antibodies of equal titer. In the third case, where the maternal serum contained moderately strong (24 units) Rh agglutinins, the infant's serum contained no Rh agglutinins but univalent Rh antibodies of 2½ units. In the latter case, presumably, the maternal serum also contained Rh univalent antibodies of 2½ units titer, but these were masked by the Rh agglutinins. Thus, the placenta acts as a semipermeable membrane which permits the free passage of univalent antibodies but holds back bivalent antibodies.

In the four control cases* in which the maternal serum contained no Rh antibodies, the infants, though Rh-positive, were not erythroblastotic and the red cells showed no evidence of coating.

Of considerable importance is the correlation between Rh antibody titer and the severity of the disease in the fetus and infant. Where the maternal serum contains univalent antibodies which readily pass the placental barrier, a good correlation is to be expected. On the other hand, if the maternal serum contains only bivalent antibodies, the titer may be less important than other factors such as an accidental defect in the placental barrier or increased intrauterine pressure causing the antibodies to enter the fetal circulation.[6] These expectations have been fulfilled in our series of cases. When the maternal serum contains potent blocking antibodies, the Rh-positive fetus is invariably delivered as a macerated stillbirth; such cases were not included in Table II because the fetal blood in these cases was not suitable for examination. It is significant that of the cases listed in Table II the four babies that died in spite of treatment came from the mothers with the highest titers of univalent antibodies. Similar observations showing the correlation between quality and quantity of antibody and prognosis have been made by Sacks and associates[17] and by Torregrosa,[18] in conformity with Wiener's theory,[6] and more recently by Davidsohn.[19]

SUMMARY

Of the various methods of demonstrating univalent Rh antibodies, the blocking test is the least sensitive, the plasma conglutination test is about ten times as sensitive as the blocking test, and the albumin-plasma conglutination test is about four times as sensitive as the plasma conglutination method. The antiglobulin technique is about equal in sensitivity to the plasma conglutination technique.

When the maternal serum contains univalent Rh antibodies, these antibodies pass through the placenta and coat the infant's Rh-positive red cells, as can be demonstrated by the blocking, conglutination, and antiglobulin techniques. The higher the titer of the univalent antibody in the maternal serum, the more completely are the fetal red cells coated and the more likely is the fetal plasma to contain free Rh antibody.

When the maternal serum contains bivalent antibodies, the fetal red cells will not be coated unless the maternal serum also contains univalent Rh antibodies.

When an Rh-negative woman with univalent Rh antibodies has an Rh-negative baby, the baby will not be erythroblastotic but its plasma will contain univalent Rh antibodies equal in titer to that of the maternal plasma at the time of birth.

Rh agglutinins usually are not demonstrable in the plasma of an Rh-negative infant even when the maternal serum contains a high titer of these antibodies.

*Many more such cases have been studied, but four are sufficient for purposes of illustration.

The placenta acts as a semipermeable membrane which permits the passage of univalent antibodies but holds back bivalent antibodies.

Infants of nonsensitized Rh-negative women are not erythroblastotic provided other sensitizations are also excluded, for example A-B sensitization.

Where the maternal serum contains univalent Rh antibodies, the severity of the manifestations in the erythroblastotic infant is roughly proportional to the antibody titer.

Evidence is presented suggesting the presence of a substance in fetal plasma, conglutinoid, which is adsorbed by cells sensitized by univalent antibodies but fails to clump them. Conglutinoid seems to be capable of blocking the action of conglutinin in much the same way that so-called complementoid blocks the action of complement.

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A STUDY OF CHOLINESTERASE ACTIVITY OF THE BLOOD OF PATIENTS WITH PERNICIOUS ANEMIA

LEO M. MEYER, M.D., ARTHUR SAWITSKY, M.D.,* NORTON D. RITZ, M.D., AND HOWARD M. FITCH, PH.D.
BROOKLYN, N. Y.

IN 1940, Sabine[1] reported red cell esterase rises with concomitant plasma and whole blood esterase reduction in two phlebotomized dogs. In a study[1] of fifteen patients with various types of anemias she reported low plasma esterase activity in debilitated and comatose states. The red cell esterase activity in these patients did not parallel that of the plasma. In general, the red cell and plasma activities were lower in those patients with hematocrits less than 30 per cent and there was normal activity where the hematocrit was above this value. Three patients with pernicious anemia in relapse receiving liver therapy were studied during their recovery. Initially, the whole blood, red cell, and plasma esterase activities were low. During treatment the cell esterase returned to normal levels in four to nine days. This was followed by a further increase in activity and a decline to normal levels in about six weeks. The plasma esterase responded much more slowly, rising to normal in about six weeks.

Previously, Antopol[2] and associates reported a general lowering of serum esterase in patients with secondary anemias and acute hemolytic anemia. Hall and Lucas[3] found no correlation between the serum esterase activity and the hemoglobin concentration, red cell and white cell counts. Ginsberg, Kohn, and Necheles[4] found that washed pus cells had no esterase activity. Milhorat[5] reported lowered serum esterase in patients with leucemia. He noted no correlation between the serum esterase and the hemoglobin, or between the serum esterase and the red cell count of the peripheral blood. There is general agreement that the plasma or serum cholinesterase activity is decreased in debilitated states[1, 5-7] and in liver and biliary tract diseases.[2, 6, 7] Low normal values are found in association with acute infections.[3] Hall and Lucas[3] could not demonstrate any relationship between the serum esterase activity and age, sex, diet, body activity, heart rate, or blood pressure in normal and pathologic sera. The role of cholinesterase in myasthenia gravis and the neuromyopathies is not clear. In these diseases the red blood cell esterase is reported to be normal, while the plasma esterase is generally reduced.[7, 8]

Since 1944 a series of articles has been published by Davis and coworkers[10-14] concerning the experimental production of a hyperchromic anemia in dogs which responds to antipernicious anemia therapy. Interestingly enough, one report[13] contains a description of nervous system changes similar to the neuropathologic changes noted in human pernicious anemia. Concomitant

From the Medical Service of Kings County Hospital, and the Department of Therapeutics, New York University College of Medicine, New York City.

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*Lederle Fellow in hematology.

with the production of the anemias, the cholinesterase activity of the dog sera fell to a low value. With the institution of antipernicious anemia therapy, the red cell count, hemoglobin and esterase activity of the serum returned to normal levels. In 1946, Davis[11, 12, 14] reported that folic acid could be used in place of liver extract. He also stated that folic acid administered to normal human subjects increased the serum esterase activity significantly within five hours. During the same year, he observed an increase in an acetylcholine-like substance in the blood of patients with pernicious anemia which returned to normal levels after antipernicious anemia therapy.[12] At this time he noted that the serum esterase activity paralleled the rising red cell count.

This report is concerned with the study of the cholinesterase activity of whole blood, red cells, and plasma in patients with pernicious anemia treated with folic acid and with combined folic acid and liver extract therapy.

METHODS

Seven patients with pernicious anemia in relapse were studied. The diagnosis was made on the basis of the peripheral blood picture, a megaloblastic bone marrow, the absence of free hydrochloric acid in the stomach, the absence of gastrointestinal disease after fluoroscopic and x-ray study, and guaiac-negative stools. A reticulocytosis following adequate antipernicious anemia therapy was always noted.

Base lines for reticulocytes, hemoglobin, red cells, and cholinesterase activity were established before treatment was given. Cholinesterase activity was determined in the oxalated venous blood before and during the course of therapy.

Cholinesterase activity was measured by the method described in a previous report.[15]

CASE REPORTS

CASE 1.—M. F., a 70-year-old white woman, known to have pernicious anemia of several years' duration, sought treatment because of weakness, dyspnea, and sore tongue. Because of liver sensitivity she had had no therapy for the previous four months. Prior to that, treatment had been intermittent and irregular. Physical examination revealed a sore red tongue. The liver and spleen were not palpable. The gait was normal and the Romberg was negative. The knee jerk reflex was hyperactive and the Babinski sign was bilaterally positive. Position and vibratory sense were normal.

Laboratory Data.—The stools were negative for occult blood. Gastric analysis revealed achlorhydria after histamine. The cephalin cholesterol flocculation was 1 plus and the icterus index was 10. The blood urea nitrogen was 15 mg. and the blood sugar was 90 mg., per 100 ml. of blood. The urine was normal. The bone marrow was not studied. Peripheral blood counts are shown in Table I.

The patient was given 20 mg. of folic acid by mouth daily in the form of an elixir. Seventy-two hours later improvement of appetite and general status was noted. In one week the tongue symptoms were relieved. After three weeks, neurological examination disclosed no abnormalities.

Before treatment the cholinesterase activity of the blood was low (about one-half the normal range). On the fourth day of therapy there was a simultaneous rise in the reticulocytes and an increase of the mean cell volume and mean cell esterase. Reticulocytosis was maintained for sixteen days. The corpuscular esterase activity reached a peak of 12.0 ($\times 10^{-10}$) units (normal, 6.19 to 8.69 [$\times 10^{-10}$] units) on the twentieth day of treatment and returned to normal levels about the thirty-sixth day. The mean corpuscular volume tended to parallel the reticulocyte curve, reaching a peak on the eighth day and returning to normal levels on the thirty-sixth day. The plasma esterase values remained constant throughout.

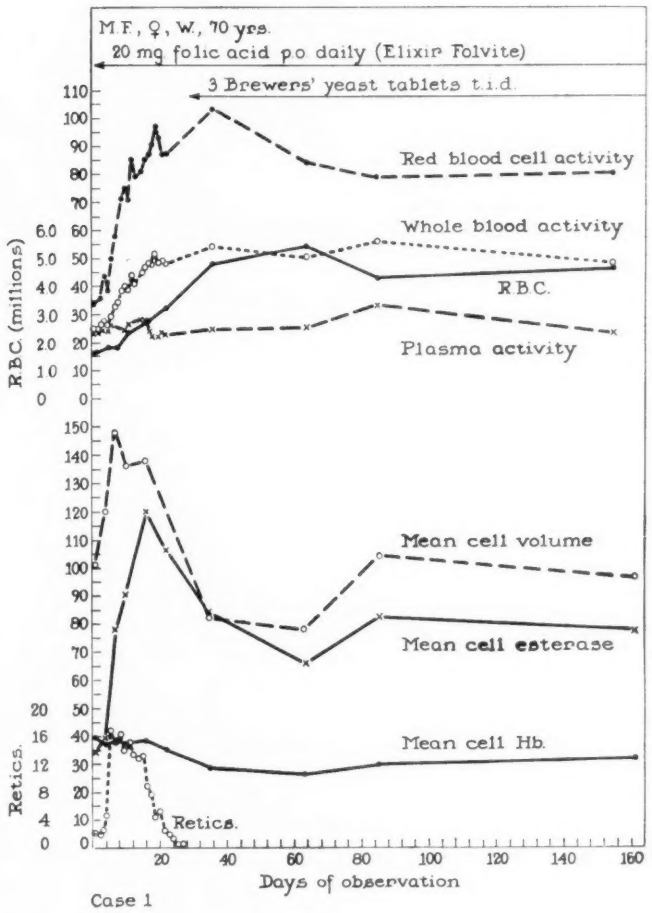

Fig. 1.

The erythrocyte count was not significantly increased until the eleventh day. Mean corpuscular hemoglobin gradually decreased, reaching the normal range about the thirty-sixth day.

CASE 2.—M. P., a 73-year-old Negro woman, was admitted to Kings County Hospital complaining of weakness and weight loss of three months' duration. There was no history of bleeding. Physical examination revealed an extremely pale, malnourished Negro woman. She was disoriented, incoherent, and belligerent. The only other positive finding was bilaterally hyperactive knee jerk reflexes.

Laboratory Data.—The stools were negative for blood and gastric analysis revealed histamine refractory achlorhydria. A gastrointestinal series and a barium enema study disclosed no abnormalities. The total blood proteins were 5.8 Gm. per 100 milliliters. The other blood findings per 100 ml. were: calcium, 9.8 mg.; phosphorus, 3.5 mg.; urea nitrogen, 15 mg.; blood sugar, 86 milligrams. The alkaline phosphatase was 4.1 units and the icterus index, 1.9. The bone marrow showed 30 per cent megaloblasts. The peripheral blood picture is shown in Table I.

Treatment of the patient was begun and 1 unit of liver extract combined with 0.3 mg. of folic acid was given intramuscularly daily. For the first seventy-two hours sedation was required. A diarrhea was noted which persisted for the following ten days. After ninety-six hours the appetite was improved and general clinical improvement was noted. The reticulo-

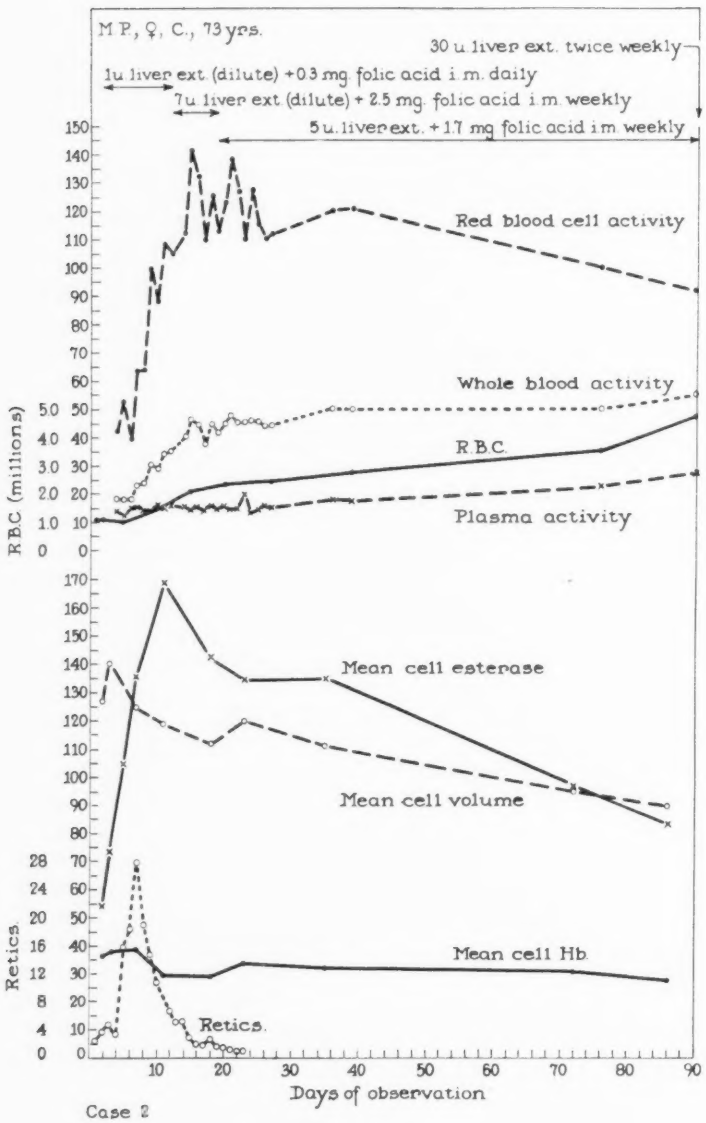
M P, ♀, C., 73 yrs.
30 u. liver ext. twice weekly
1u. liver ext. (dilute) + 0.3 mg. folic acid i.m. daily
7u. liver ext. (dilute) + 2.5 mg. folic acid i.m. weekly
5 u. liver ext. + 1.7 mg. folic acid i.m. weekly
Red blood cell activity
Whole blood activity
R.B.C.
Plasma activity
RBC (millions)
Mean cell esterase
Mean cell volume
Mean cell Hb
Retics.
Retics
Days of observation
Case 2

Fig. 2.

cyte response and the cholinesterase activity are shown in Fig. 2. The patient made an uneventful recovery and on discharge was instructed to take 30 units of liver extract twice weekly.

The initial esterase values were obtained on the second day of therapy and were low (about one-half the average normal value). On the fifth day significant increases in red cell esterase and in the number of reticulocytes were noted. The erythrocyte count rose and the mean corpuscular volume and hemoglobin started to fall. The mean cell esterase activity continued to rise, reaching a peak on the eleventh day. The reticulocyte curve returned to normal about the eighteenth day. The corpuscular esterase fell more slowly; it was abnormally high after 36 days, but returned to normal by the seventieth day. The plasma esterase remained constant for twenty-five days, then rose slowly, paralleling the erythrocyte increase.

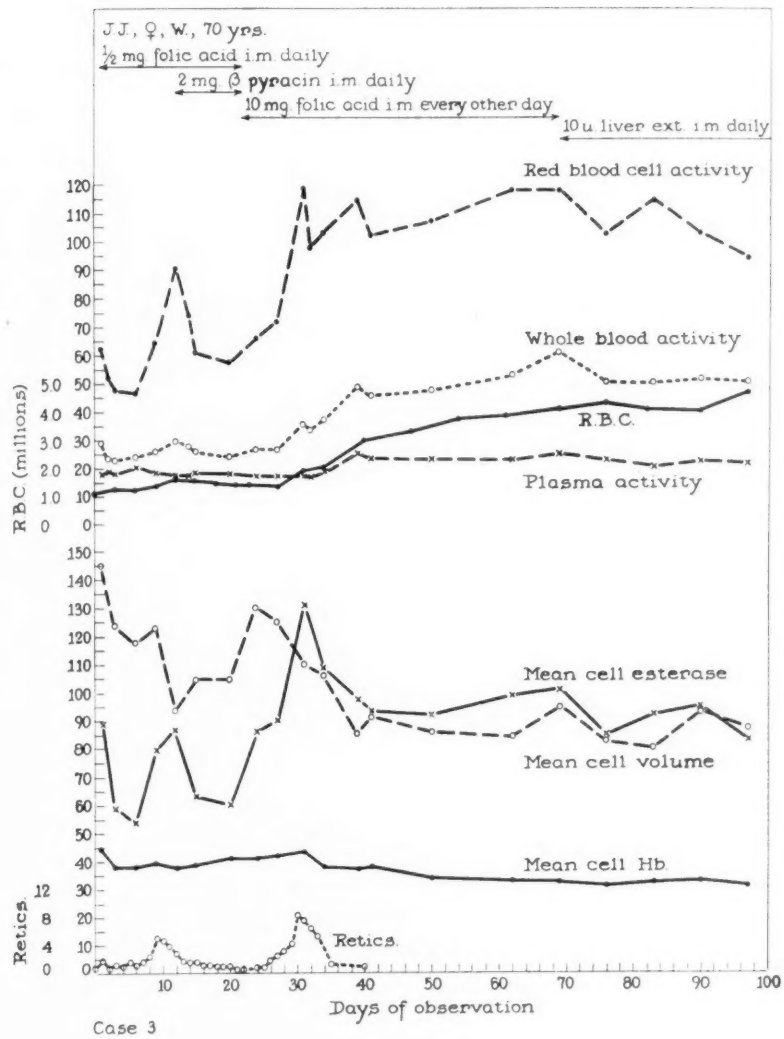

Fig. 3.

CASE 3.*—J. J., a 70-year-old white woman, was admitted to the Kings County Hospital complaining of progressive dizziness and tinnitus of four months' duration. One week before admission, weakness, blood-tinged diarrhea, dysuria, and polyuria were noted. A history of inadequate diet and the daily consumption of a pint of liquor for the past thirty years was obtained. On physical examination the only positive findings were a blowing apical systolic murmur, moderate right costovertebral angle tenderness, and external hemorrhoids.

Laboratory Data.—A routine blood count revealed macrocytic hyperchromic anemia and 2 per cent megaloblasts in the peripheral blood. Bone marrow aspiration was confirmatory and a megaloblastosis of 32.5 per cent was noted. Gastric analysis was done and a histamine refractory achlorhydria was found. The chest x-ray and gastrointestinal series disclosed no abnormalities. Urine examination showed many white blood cells with clumping. The blood chemistry was normal.

*From the Medical Service of Dr. W. Dock and Dr. R. Janet Watson, Kings County Hospital.

The patient was disorderly on admission and on the next day was disoriented and psychotic. She was given 0.5 mg. of folic acid intramuscularly daily for twelve days. At this time 2 mg. of beta-pyracin were added to the daily dose. There was no clinical response, but a reticulocytosis of 5.2 per cent was observed. After ten days the daily administration of 10 mg. of folic acid intramuscularly was started and a clinical and hematologic response followed. Nineteen days later, because of continued disordered sensorium, therapy was changed to the daily administration of 10 units of liver extract by the intramuscular route. After eighteen days of liver therapy the sensorium cleared and the patient made an uneventful recovery.

The initial esterase values were low, averaging 5 units of red cell esterase, 6 ($\times 10^{-10}$) units of corpuscular esterase, and 1.8 units of plasma esterase. Following inadequate therapy with folic acid and beta-pyracin, red cell and corpuscular esterase activities rose to a peak of about 9 units (in normal range) on the twelfth day and then fell rapidly to 6 units on the twenty-third day. A reticulocyte peak of 5.2 per cent was noted on the ninth day. The reticulocytes dropped to 1.8 per cent on the eleventh day and remained at about 1 per cent until seven days after folic acid in 10 mg. doses was given, when another peak of 8.2 per cent was noted. The number of erythrocytes rose slightly during the first period and then fell off to pretreatment levels until effective folic acid therapy was given. Nine days later the red cell and corpuscular esterase rose sharply to a peak of 11.7 units and 13.0 ($\times 10^{-10}$) units, respectively. The plasma esterase remained relatively constant throughout.

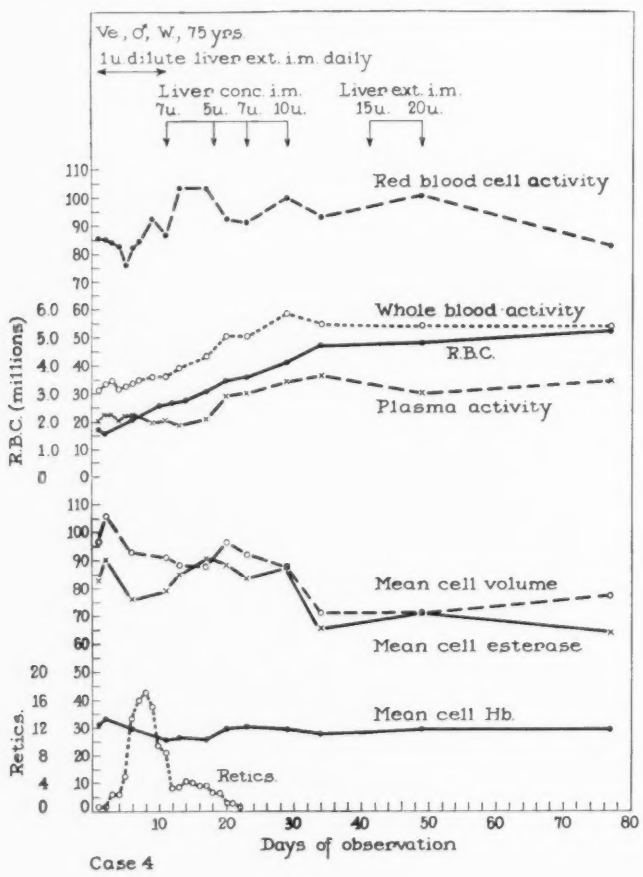

Fig. 4.

CASE 4.—Ve., a 75-year-old white man who was seen by one of us (L. M. M.), complained of weakness and loss of appetite during a period of one year. Six months previously he had been given a transfusion for "anemia" but no other therapy. Physical and neurological

examinations disclosed no abnormalities. A gastrointestinal series was normal and there was no blood in the stoools. The blood findings were: nonprotein nitrogen, 27 mg., and the fasting sugar, 95 mg. per 100 ml.; icterus index, 8. The patient was treated with 1 unit of liver extract daily for ten days and then single injections of liver extract in equivalent unit dosage were given at approximately weekly intervals.

Initially the red blood cell and mean corpuscular esterase activities were within normal limits. The plasma activity was only slightly decreased. Following liver therapy there was a small increase in the erythrocyte esterase activity; a maximal value was attained on the sixteenth day. This value was sustained for an equal period of time and then fell to a lower plateau within the normal range. The plasma esterase rose to normal on the nineteenth

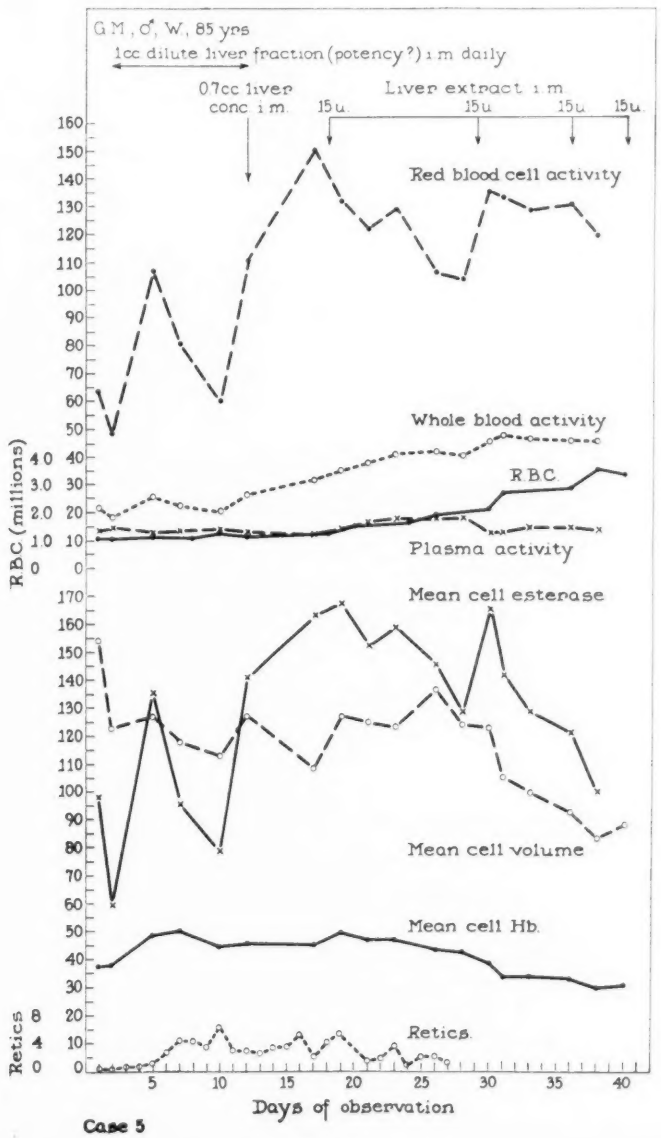

Fig. 5.

day. On the fourth day after therapy, a reticulocytosis was noted which reached a peak of 17.2 per cent on the seventh day. The erythrocyte count rose steadily as is shown in Table II.

CASE 5.—G. M., an 85-year-old white man, was admitted to the Psychiatric Pavilion of Kings County Hospital because he was paranoid, abusive, hyperemotional, and unpredictable in his actions. He was transferred to the Medical Service when a severe anemia was found. Physical examination disclosed a well-nourished man with a lemon-yellow tint to the skin, icteric sclerae, and pale conjunctivae. Neuropsychiatric examination elicited gross memory defect, lack of insight, and disorientation as to time and place.

Laboratory Data.—The urine analysis disclosed no abnormalities. Histamine refractory achlorhydria was found. The stools were free of blood. The cephalin flocculation was negative and blood cholesterol was 153 mg. per 100 milliliters. A bone marrow aspiration revealed sheets of megaloblasts. The peripheral blood findings are shown in Table I.

The patient was difficult to manage and required restraint. He was treated with 1 unit of liver extract intramuscularly daily, and a mild clinical but no hematologic response was noted. Ten days later he was given 10 units of liver extract. After six days he received fifteen units of liver daily intramuscularly. There was clinical improvement, but a submaximal reticulocytosis was obtained. The patient's mental status remained impaired and on the fortieth day of observation the patient was transferred to the psychiatric ward.

The initial mean corpuscular esterase and red cell esterase activities were in the normal and low normal range, but the plasma activity was about one-half of normal values. Three days after the beginning of liver extract therapy the reticulocyte count rose, reaching a plateau of 4 to 6 per cent which was sustained for twenty-two days. The red cell esterase increased rapidly to 13.5 ($\times 10^{-10}$) units on the third day and then fell abruptly to the initial levels on the eighth day. There was a secondary rise to 14 ($\times 10^{-10}$) units two days later, with a peak of 16.5 ($\times 10^{-10}$) units on the sixteenth day. The red cell esterase values then slowly fell toward lower but normal levels. The plasma activity was constant and low throughout the period of observation. The mean cell volume was above normal for thirty days and then returned to normal. The red cell count remained stationary at about 1,000,000 per c.mm. for sixteen days and then rose slowly toward normal. This erythrocyte response was observed six days after the second red cell esterase rise.

CASE 6.—A. P., a 50-year-old white woman known to have pernicious anemia, was admitted to Kings County Hospital for weakness and pallor of six months' duration. She had been treated fifteen months previously for pernicious anemia in relapse and had responded well to 0.5 units of liver and 10 mg. of folic acid intramuscularly daily. She stopped all medication eight months before the present admission. Physical examination was negative.

Laboratory Data.—A gastrointestinal series was negative and the stools were negative for blood. The cephalin flocculation was 1 plus, the alkaline phosphatase, 4 units, and the icterus index, 6. The blood total protein was 6.2 Gm. per 100 milliliters. The urinary urobilinogen was positive to a dilution of 1:10.

The patient was treated with an experimental liver fraction without hematologic or clinical response. After five days, oral treatment with 1 unit of liver and 5 mg. of folic acid was started. Within forty-eight hours there was marked clinical improvement. The patient went on to make an uneventful recovery. The peripheral blood picture and the cholinesterase response are shown in Table II.

The initial red cell esterase values were only slightly below the normal range and rose rapidly to normal following the injection of an experimental liver fraction. There was no reticulocytosis and no erythrocyte rise was noted during this period. Three days after adequate therapy with liver and folic acid was instituted a reticulocytosis ensued. A peak of 24 per cent occurred on the seventh day. On the thirteenth day, following combined oral liver and folic acid, the mean cell cholinesterase activity rose above normal and reached a peak of 10.8 ($\times 10^{-10}$) units on the sixteenth day. The red cell esterase gradually fell to normal values by the forty-second day. The erythrocyte count remained unchanged until eleven days

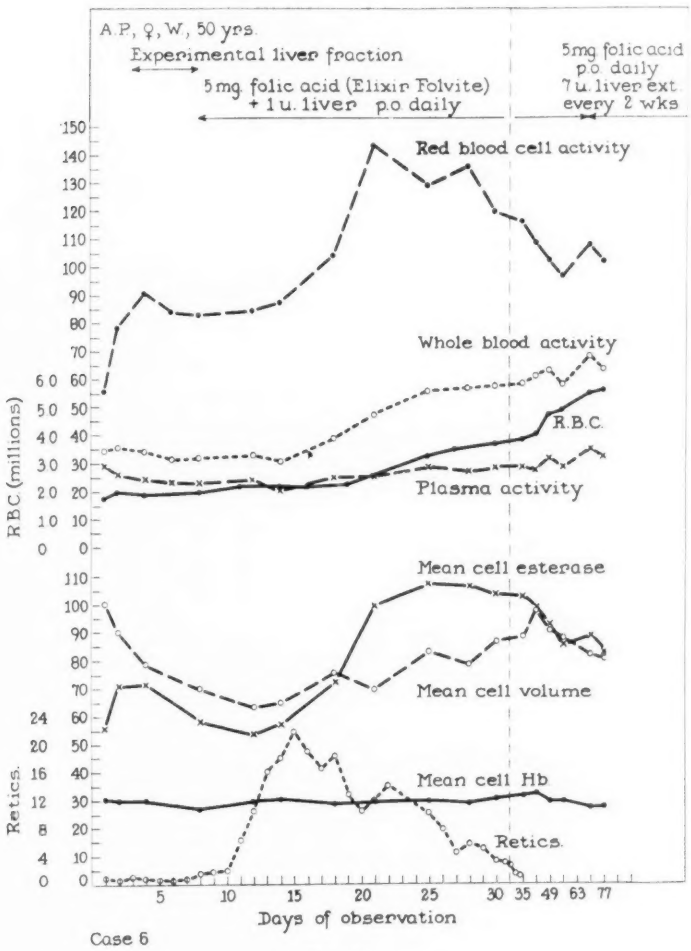

Fig. 6.

after combined therapy was given and then slowly rose to normal levels. There was an equivocal rise of the plasma activity seventeen days after the institution of combined therapy.

CASE 7.*—M. O., a 56-year-old white man, was admitted to Metropolitan Hospital for anosmia of one year's duration, anorexia, weakness, dyspnea of effort, and dysphagia of four months' duration. Diarrhea for three weeks prior to admission was also noted. The only significant findings on physical examination were marked pallor, slight icterus of the sclerae, a smooth clean tongue, and malnutrition.

Laboratory Data.—The peripheral blood revealed the presence of a macrocytic hyperchromic anemia. A bone marrow aspiration was "compatible with pernicious anemia or sprue." The chest x-ray, a gastrointestinal series, and the electrocardiogram disclosed no abnormalities. The urine showed a 1 plus test for albumin. The blood urea nitrogen was 15 mg., and the blood sugar, 115 mg. per 100 milliliters. The value for total blood proteins was 6.7 Gm. per 100 c.c., with an A/G ratio of 2. The total blood cholesterol was 200 mg. per 100 c.c., of which 35 per cent were esters. The icterus index was 7 and the van den Bergh

*From the Medical Service of Dr. Thomas H. McGavack, Metropolitan Hospital, Welfare Island, N. Y.

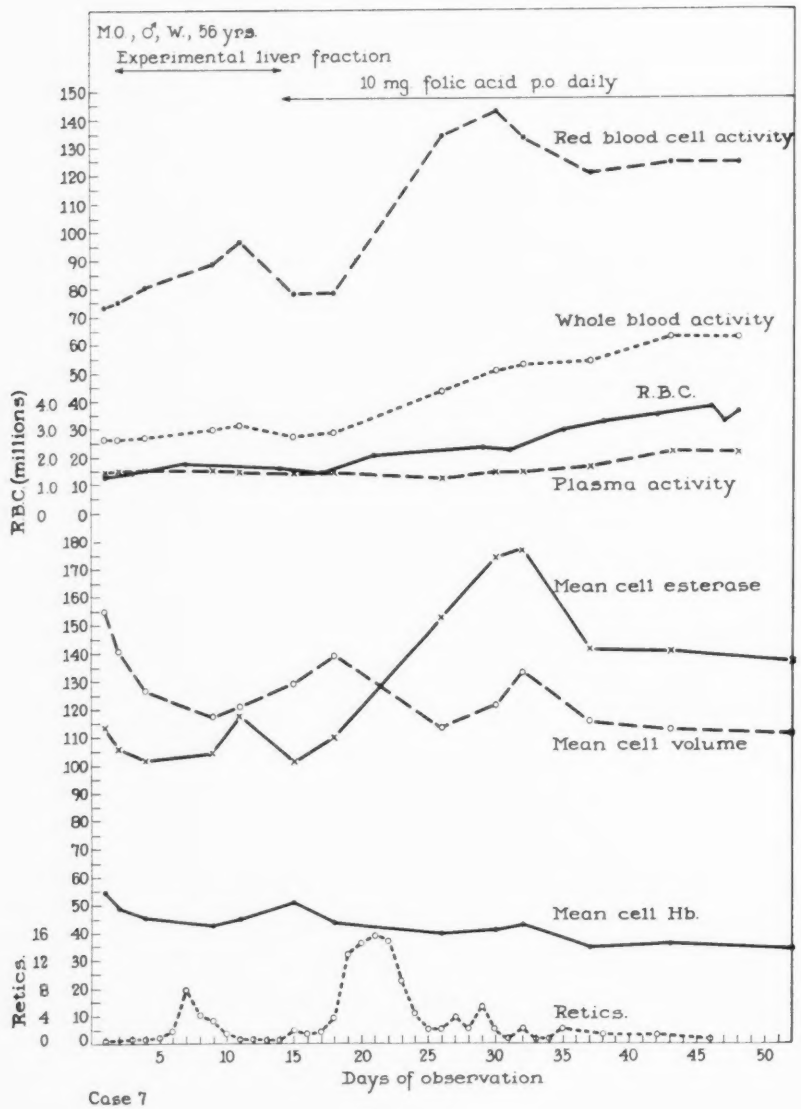

Fig. 7.

reaction was negative. The cephalin flocculation was negative and the alkaline phosphatase was 1.9 units.

The patient was treated with a special experimental liver fraction without a clinical response. He was then given 10 mg. of folic acid daily by mouth; this treatment was followed by an uneventful clinical and hematologic remission.

Initial red cell and mean corpuscular esterase values were in the normal range. The plasma esterase activity was about one-half the mean normal value. Following the injection of an experimental liver fraction there was definite but unsustained increase in erythrocyte cholinesterase activity. A submaximal reticulocytosis of 7.8 per cent was also noted. There was an equivocal transient rise in the red blood count. When all the values had returned to pretreatment levels, treatment with folic acid in daily oral doses of 10 mg. was initiated. This

was followed by a rapid increase in all values except that of plasma cholinesterase; the latter did not change for sixteen days and then rose in a manner paralleling that of the red cell count.

RESULTS

In ten patients with pernicious anemia in relapse the mean esterase activity of the red blood cells was 5.90 ($\times 10^{-10}$) units as compared with 7.55 ($\times 10^{-10}$) units in normal subjects. In four patients it was within the normal range of 6.19 to 8.69 ($\times 10^{-10}$) units. The plasma levels were low with an average activity of 1.60 units as compared with 3.31 units in the normal. Eight of the plasma determinations in the patients with pernicious anemia were within the low normal range (Table I). Three to nine days after beginning effective antipernicious anemia therapy there was a rapid rise in red blood cell esterase. The red cell activity rose to above normal value, remained high for thirty-five to eight-five days, and declined to the average range. The plasma esterase activity remained unchanged at the initial low levels and did not rise until eighteen to thirty days after the onset of effective therapy. The slope of the plasma rise tended to parallel the slope of the erythrocyte rise as both returned to normal.

TABLE I. PERNICIOUS ANEMIA IN RELAPSE

SAMPLE	WHOLE BLOOD CHE (UNITS/ML.)	PLASMA CHE (UNITS/ML.)	R.B.C. CHE (UNITS/ML.)	HB. (GM. PER 100 ML.)	R.B.C. ($\times 10^6$ PER MM.^{3})	M.C.V. (CU. μ)	CELL CHE (UNITS $\times 10^{-10}$ PER CELL)
M. F.	2.50	2.32	3.35	6.4	1.68	101	3.38
M. P.	1.84	1.44	4.29	4.0	1.10	127	5.44
Ja.	2.28	1.81	4.75	4.9	1.29	124	5.90
Ve.	3.12	2.00	8.59	5.5	1.75	97	8.33
G. M.	1.82	1.48	4.87	4.0	1.06	122.5	5.96
Kw.	1.11	0.54	6.20	3.4	0.76	132	8.15
Kw.	1.35	0.98	4.36	6.5	1.30	85	3.69
Cu.	1.60	1.14	5.70	3.0	0.72	137	7.90
A. P.	3.45	2.97	5.61	5.5	1.80	100	5.61
M. O.	2.63	1.45	7.35	7.0	1.29	155	11.39
Fi.	2.62	1.50	5.81	7.0	2.55	102	5.93
Mean	2.28	1.48	5.61	5.50	1.29	122.5	5.90

Whole blood ChE, cholinesterase activity in 1 ml. of whole blood; Plasma ChE, cholinesterase activity in 1 ml. of plasma; R. B. C. ChE, cholinesterase activity in 1 ml. of red blood cells; M. C. V., mean corpuscular volume in cubic microns; Cell ChE, mean corpuscular esterase activity in units per cell ($\times 10^{-10}$).

There was no correlation between the initial red cell esterase activity, the red cell count, or the hemoglobin concentration. The mean corpuscular esterase was similarly unrelated to the mean corpuscular volume. There was an apparent relationship between the initial plasma esterase and the patient's general condition. The plasma esterase was not dependent, however, upon the initial red cell count or the hemoglobin concentration.

Following adequate antipernicious anemia therapy, and in patients maintained on adequate therapy, the mean red cell enzyme activity was 8.31 ($\times 10^{-10}$) units (normal, 7.55 [$\times 10^{-10}$]). The plasma level in these patients was 2.63 as compared with 3.31 in normal subjects. The mean hemoglobin concentration was 14.0 Gm. per 100 ml. of blood, and the mean erythrocyte count was 4,600,000 per c.mm. (Table II).

TABLE II. PERNICIOUS ANEMIA IN REMISSION

SAMPLE	WHOLE BLOOD CHE (UNITS/ ML.)	PLASMA CHE (UNITS/ ML.)	R.B.C. CHE (UNITS/ ML.)	HB. (GM. PER 100 ML.)	R.B.C. ($\times 10^6$ PER MM.^{3})	M.C.V. (CU. μ)	CELL CHE (UNITS $\times 10^{-10}$ PER CELL)
M. F.	4.80	2.30	8.00	14.5	4.55	96.5	7.73
M. P.	5.58	2.80	9.26	13.3	4.80	89.5	8.31
Ja.	5.00	2.10	9.35	14.0	4.60	87	8.23
Ve.	5.37	3.43	8.28	15.1	5.20	77	6.37
G. M.	4.64	1.48	12.00	10.5	3.60	83.5	10.00
Ham.	6.18	3.91	9.31	14.0	4.60	91.5	8.56
J. A.	4.92	2.66	9.11	12.2	4.40	79.5	7.25
A. P.	5.80	2.86	9.70	14.5	4.90	87.7	8.51
M. O.	6.30	2.16	12.50	12.0	3.60	111	13.70
Mean	5.37	2.66	9.31	14.0	4.60	87.7	8.31

Whole blood ChE, cholinesterase activity in 1 ml. of whole blood; Plasma ChE, cholinesterase activity in 1 ml. of plasma; R. B. C. ChE, cholinesterase activity in 1 ml. of red blood cells; M. C. V., mean corpuscular volume in cubic microns; Cell ChE, mean corpuscular esterase activity in units per cell ($\times 10^{-10}$).

Four patients (Cases 3, 5, 6, and 7) were given preliminary suboptimal therapy with either folic acid or experimental liver fractions of varying potency without causing a significant rise in the erythrocyte counts. In three of these patients (Cases 3, 5, and 7) small reticulocyte peaks of less than 8 per cent were observed nine, five, and six days, respectively, after injection of the extract. A rapid unsustained peak to normal values of the mean cell esterase was noted nine, four, and ten days, respectively, after the fraction was given. One patient (Case 6) had no reticulocytosis, and no effect on cholinesterase values was seen.

DISCUSSION

It would appear that in the normal individual the cholinesterase activity of the whole blood, red cells, and plasma remains constant within a narrow range from day to day and month to month.[15] Patients with pernicious anemia in relapse exhibit low whole blood, red cell, and plasma esterase activity. When therapy is instituted the esterase activity of whole blood and red cells increases rapidly. This increase is shown to be due to an increase in the mean red cell esterase activity and does not reflect an erythrocyte rise which it invariably precedes. The plasma cholinesterase remains constant during this period. In four patients (Cases 1, 2, 3, and 7) the initial rapid red cell cholinesterase rise occurred at the same time that a reticulocytosis was noted. In cases 4 and 6 the reticulocytosis preceded the red cell esterase rise. In Case 5 the reticulocytosis continued even when the red cell esterase activity was falling. Where subeffective therapy was given, minimal reticulocytic response was associated with significant red cell esterase rise. Thus, a direct relationship between red cell esterase and reticulocytosis could not be established. In Cases 2, 3, 5, 6, and 7 the cell cholinesterase was not dependent on the mean cell volume. In two patients (Cases 1 and 4) the red cell esterase curves closely followed the curve of the mean cell volume.

Apparently patients with pernicious anemia in relapse have erythrocytes that are abnormally low in cholinesterase activity, and plasma that exhibits a subnormal esterase activity. The administration of liver extract or folic acid,

parenterally or orally, is immediately followed by an increase in the cholinesterase activity of the red cells. This increase is most frequently carried to levels greater than those found in the red cells of normal subjects. Following a variable period of time (about five to ten weeks), the red cell activity declines to and remains within the normal range. At this time a rise in plasma esterase is noted.

Sabine[1] postulated a normal mechanism for maintaining a high esterase in the mature cell of the anemic patient and the failure of the mechanism in the patient whose marrow is incapable of responding. She further postulated a specific defect in pernicious anemia with an "inability to put out cholinesterase in adequate amounts in such cells as the marrow produces." Davis[10-12] believes that relapse in pernicious anemia is related to the increased activity of acetylcholine. He believes that this nereased concentraton produces a dilatation of the vascular network of the bone marrow with an increase in marrow oxygen tension. With this increase in oxygen tension an inhibition of the maturaton of cells of the erythroid series occurs.

We have accumulated data[16] in various other anemias. It is our experience that pernicious anemia alone shows the consistently low cholinesterase activities we have described, with the possible exception of myeloid leucemia. Other anemias with similar low hemoglobin and erythrocyte counts are associated with normal or above normal red cell esterase activity. The plasma activity in these cases is dependent upon the patients' general condition being as low as or lower than the levels herein reported. Reticulocyte counts in these cases have revealed no consistent trend.

SUMMARY

Observations on seven patients with pernicious anemia in relapse are reported. The cholinesterase activity of whole blood, red cells, and plasma has been followed during their return to remission.

The cholinesterase activity of whole blood, red cells, and plasma in pernicious anemia patients in relapse is below normal levels.

With therapy, the cholinesterase activity of the red cell is the first to rise. This rise in activity is not related to the reticulocytosis, the mean cell volume, or the increase in the number of red cells.

The plasma activity remains constant until the values for red cell esterase have become abnormally high and then have returned to normal levels.

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A STUDY OF CHOLINESTERASE ACTIVITY IN THE BLOOD OF NORMAL SUBJECTS

ARTHUR SAWITSKY, M.D.,* HOWARD M. FITCH, PH.D., AND LEO M. MEYER, M.D.
NEW YORK, N. Y.

DALE,[1] in 1914, postulated the presence of a blood esterase which would inactivate acetylcholine. Loewi,[2] working with the isolated frog heart, demonstrated that stimulation of the vagus liberated a substance which itself was capable of producing the effect previously thought to be due to action of the nerve. Loewi and Navratil[3] showed that this vagus substance was an ester of choline, probably acetylcholine. These authors also found that aqueous extracts of frog heart inactivated acetylcholine. Because these extracts acted in an enzyme-like manner, they called the active principle an esterase. In 1930, Engelhart and Loewi[4] and, independently, Matthes[5] conclusively demonstrated the enzymic nature of the destructive agent. Three years later, Stedman, Stedman, and Easson[6] presented evidence of the presence of cholinesterase in the blood serum of the horse. In 1935, Stedman and Stedman[7] reported that sera from different species differed widely with respect to the content of cholinesterase. These authors found that the erythrocytes of various species of animals also contained appreciable amounts of enzyme. By this time, a host of papers had appeared and various phases and characteristics of the enzyme were reported in the literature. Almost all of the earlier work was based on the enzyme present in serum or plasma.

Plattner and Galehr,[8] using a biologic assay method, found that the cholinesterase activity of erythrocytes was greater than that of serum. Matthes[5] confirmed this finding. Stedman and Stedman[7] showed in a series of experiments using a physicochemical technique that sera of different species differed widely with respect to the cholinesterase content and that the erythrocytes of various species contained appreciable amounts of enzyme. Hall and Lucas[9] studied normal and pathologic human sera and concluded that age, sex, activity, diet, heart rate, and blood pressure were without influence on the serum esterase. They reported that the range of serum esterase values for any individual was constant, but that this might vary widely from individual to individual. In 1940, Sabine[10] showed that the cholinesterase activity of erythrocytes was greater than that of plasma in both normal and pathologic subjects. Alles and Hawes[11] confirmed her findings.

Brauer and Root[12] recently demonstrated a correlation between liver cholinesterase and the serum esterase of the dog. Similarly, it has been shown[13] that the rate of human serum esterase regeneration is significantly lower in patients with liver disease than in the normal subject. Cohn and co-workers[14] have found that plasma esterase activity is largely concentrated in plasma fraction IV-4. This plasma fraction is rich in alpha and beta globulins of low lipid

From the Department of Therapeutics, New York University, College of Medicine.
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*Lederle Fellow in hematology.

content. Mendel and associates,[15, 16] on the basis of substrate concentrations and substrate specificity, postulate two different cholinesterases in the body—one a specific of true esterase found in red blood cells and brain tissue, the other a nonspecific or pseudoesterase found in serum and plasma. Nachmansohn and Rothenberg[17, 18] have inclined toward the view that specificity is relative and that tissues containing the specific esterase split acetylcholine at a higher rate than they split other esters. Bodansky[19] speaks of a "family" of cholinesterases whose members resemble one another in some characteristics and differ in others. Thus, within one species the enzyme differs in certain respects from tissue to tissue; and in a given tissue, from species to species.

METHODS

The plasma and red cell cholinesterase activities of random blood samples taken from nine male and six female subjects were determined. Serial samples were tested at monthly intervals in three of these subjects. Oxalated venous blood was drawn from a vein in the antecubital fossa. The hematocrit (Wintrobe) was determined and the samples separated into whole blood and plasma. A complete capillary blood count was done at this time. Random reticulocyte counts were done in five subjects.

The cholinesterase activity was measured in units representing the number of milliliters of 0.02N sodium hydroxide necessary to neutralize the acetic acid liberated from 100 ml. of 0.012M acetylcholine bromide solution by 1 ml. of whole blood or plasma in a twenty-minute period at a pH of 7.4 ± 0.05. The temperature was held constant at 37.5° C. Red cell esterase activity was calculated from the whole blood activity, plasma activity, and the hematocrit value. A correction factor applied to all esterase values was derived from a determination of the amount of acetylcholine hydrolyzed under the same conditions in the absence of blood. The mean corpuscular esterase was calculated by multiplying the red cell esterase activity by the mean corpuscular volume.

$$\text{Mean cell ChE} = \text{Red Cell ChE} \times \text{M.C.V.}$$
$$\text{Units/cell} = \text{Units/ml.} \times \text{ml./cell}$$

RESULTS (SEE TABLES I AND II)

TABLE I. CHOLINESTERASE ACTIVITY OF NORMAL HUMAN SUBJECTS

SAMPLE	WHOLE BLOOD CHE (UNITS/ML.)	PLASMA CHE (UNITS/ML.)	R.B.C. CHE (UNITS/ML.)	HB. (GM. PER 100 ML.)	R.B.C. ($\times 10^6$ PER MM.^{3})	M.C.V. (CU. μ)	C.CHE (UNITS $\times$ 10^{-10} PER CELL)
A. S. ♂	6.06	3.51	9.44	15.5	5.25	82	7.74
B. S. ♀	4.46	1.50	8.55	14.6	5.05	84	7.19
L. E. ♀	5.33	2.71	9.10	15.9	5.15	80	7.28
H. R. ♂	6.71	3.33	10.34	16.1	5.75	84	8.69
B. F. ♂	5.14	2.58	8.40	15.2	5.25	84	7.06
A. J. K. ♂	5.22	1.83	8.90	16.2	5.30	91	8.10
C. R. ♂	6.62	4.14	9.64	14.3	5.10	88	8.47
J. M. ♀	5.39	3.95	7.44	14.0	4.85	85	6.32
F. K. ♀	6.65	4.92	8.76	15.4	5.20	87	7.64
A. O. ♂	6.84	4.13	10.16	17.0	5.65	80	8.12
B. Sch. ♂	4.83	2.92	7.25	14.1	5.16	85	6.19
L. G. ♀	6.76	5.00	8.52	16.2	5.30	94	8.06
P. S. ♀	5.08	3.00	7.84	15.0	4.85	89.5	7.02
S. S. ♂	5.40	2.95	8.40	15.1	5.20	86.5	7.27
H. K. ♂	5.81	3.19	8.82	15.5	5.42	85	7.55
Mean	5.40	3.19	8.76	15.4	5.20	85	7.55

Whole blood ChE, cholinesterase activity of 1 ml. whole blood; Plasma ChE, cholinesterase activity of 1 ml. plasma; R.B.C. ChE, cholinesterase activity of 1 ml. red blood cells; M.C.V., mean corpuscular volume; C. ChE, mean corpuscular cholinesterase activity, units per cell.

TABLE II. CHOLINESTERASE ACTIVITY OF NORMAL HUMAN SUBJECTS; REPEATED SAMPLINGS

SUBJECT	DATE	WHOLE BLOOD CHE (UNITS/ML.)	PLASMA CHE (UNITS/ML.)	R.B.C. CHE (UNITS/ML.)	C.CHE (UNITS/CELL)
A. S.	12/9	6.13	3.43	9.42	7.76
♂	2/5	6.06	3.51	9.44	7.74
	4/7	5.98	3.20	9.38	7.47
A. J. K.	12/9	5.22	1.83	8.90	8.10
♂	2/24	5.40	2.02	9.06	8.15
	5/23	5.50	1.97	9.06	8.05
S. S.	2/5	5.40	2.95	8.40	7.27
♂	4/7	5.52	3.01	8.46	7.26
	5/23	5.46	2.89	8.60	7.54

The cholinesterase activity of 1 ml. of red blood cells ranged from 7.25 to 10.34 units, with a mean value of 8.76 units. The mean corpuscular esterase activity varied from 6.19 to 8.69 units ($\times 10^{-10}$), with a mean value of 7.55 units ($\times 10^{-10}$). The esterase activity of 1 ml. of plasma was found to be 1.50 to 5.00 units, with a mean of 3.19 units. Activity did not seem to be influenced by sex. Although the activity varied markedly from individual to individual, within the same subject the activity remained rather constant (see Table II).

SUMMARY

Our results confirm the findings of previous workers that in the human subject cholinesterase activity of the red blood cell is greater than that of the plasma. The cholinesterase activity may vary widely from individual to individual, but tends to remain fairly constant in any one individual.

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A COMPARISON OF METHODS USED FOR THE HYDROLYSIS OF CONJUGATED URINARY ESTROGENS

JOHN T. VAN BRUGGEN, PH.D.*
ST. LOUIS, MO.

A PREVIOUS report from this laboratory[7] suggested the need for a thorough study of the methods for the hydrolysis of urinary estrogens. Gallagher[7] has reviewed a number of the factors concerned in urine hydrolysis and has pointed out that present methods are not entirely satisfactory.

In addition, the fact that most methods of urine hydrolysis have established definite time intervals of treatment indicates that acid hydrolysis of urine may be, in reality, a balance between two simultaneous reactions, namely, hydrolysis and destruction. The method giving the greatest yield of free forms, then, would be one which caused the hydrolysis of a maximum amount of conjugated forms while the destructive process was kept at a minimum. The mechanics of the reactions that cause a decrease in biologic activity of estrogen preparations subjected to acid hydrolysis are as yet unknown. Pincus and Pearlman[11] have reviewed the subject of artifacts arising from the acid treatment of androgen preparations and they point out the misconceptions that may arise from such procedures.

Good yields of free estrogens were obtained by Cohen and Marrian[2] by adjusting the pH of the urine to 1.0 with HCl, further acidifying with 3.3 ml. of 12 N HCl per 100 ml. urine, and finally, heating the sample at 120° C. for two hours. Smith and Smith[12] advocated the use of reflux hydrolysis for ten minutes of urine samples made to 15 volumes per cent with HCl and found good agreement between their method and the Cohen and Marrian procedure. Dingemanse, Laqueur, and Mühlbock[4] hydrolyzed urine with 15 ml. of 25 per cent HCl per 100 ml. urine, and at the same time extracted freed forms with a layer of benzene. Three four-hour periods of boiling with a total of 150 ml. of organic solvent were used.

Other methods of treatment have been suggested[3, 8, 9, 13] but, because of difficulties of too mild or too drastic conditions, have not been adopted generally.

Leiboff and Tamis,[9] Smith and Smith[12] and Gallagher, Peterson, Dorfman, Kenyon, and Koch[6] have used continuous extraction equipment for the extraction of hydrolyzed urine. Talbot, Butler, MacLachlan, and Jones[15] reported a procedure for the simultaneous hydrolysis and extraction of urinary 17-ketosteroids.

Before urine hydrolysis studies were undertaken, the effect of hydrolysis conditions on pure estrogens was ascertained.

This work is admittedly incomplete, but since it is not possible to continue the investigation at this time, these results are being published in the hope that they may be of interest to investigators in the field.

From the Department of Biochemistry, St. Louis University School of Medicine.

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*Present address: Department of Biochemistry, University of Oregon Medical School, Portland, Ore.

Effect of Acid Hydrolysis on Free Estrogens.—Smith and Smith[14] found there was no loss of activity of estrone, estriol, or α-estradiol when solutions of these forms were treated by their hydrolysis conditions, namely 100° C. for ten minutes with 15 volumes per cent HCl, but they found there was an appreciable destruction of estrone by heating at 100° C. for twenty minutes with 15 volumes per cent HCl.

We have confirmed the observation that prolonged boiling of pure estrogens in 15 volumes per cent HCl causes destruction. Thirty minutes of boiling of pure estrogens in distilled water made 1.6 N with HCl caused 28 per cent destruction of estriol, 34 per cent of estrone, and 34 per cent of α-estradiol. As previously reported,[5] no destruction of activity occurred when pure estrogens were hydrolyzed in an atmosphere of nitrogen and in the presence of a protective agent (1-amino-2-naphthol-4-sulfonic acid). Residual estrogenic activity was determined by bio-assay, for colorimetric determinations on these same fractions showed a wide discrepancy from bio-assay results.

Hydrolysis of Known Conjugated Estrogen.—The principal estrogen conjugate in human urine appears to be estriol glucuronide. Although estrone also may be excreted in a conjugated form (perhaps as the sulfate, as in mare urine), such a compound has not been isolated from human sources.

The conditions affecting hydrolysis of the pure estriol conjugate have not been reported previously. We have studied the behavior of this compound under three conditions listed below. Aliquots of standard solutions of sodium estriol glucuronate* were added to distilled water and further treated in the following manner. The first sample was adjusted to a pH of 1.0 with HCl and allowed to stand at room temperature for ten days. Such treatment liberated only 5 per cent of the combined form. The second sample was hydrolyzed for ten minutes at 100° C. with 15 volumes per cent HCl, under N_2 and in the presence of the previously described protective agent, and gave 99 per cent hydrolysis. The third preparation was treated like the second except that only enough HCl was added to make the solution 0.1 N. In this case only 20 per cent of the estriol was liberated.

The second and third samples show the importance of acid concentration and the necessity of heat treatment and also indicate that the acid and heat treatment of the Smith and Smith[12] procedure are effective hydrolytic agents for this conjugate in pure solution.

Hydrolysis of Pregnancy Urine.—Preliminary studies on each of five urines obtained during the fifth and sixth month of pregnancy indicated a considerable variation in results. The Smith hydrolysis and our protective hydrolysis modification of the Smith method were the chief methods employed. These studies indicated that nitrogen and the protective agent in a few cases gave higher amounts of freed estrogens. As Doisy has previously reported,[5] in one of the urines tried, the potency of an aliquot of a twenty-four hour specimen was doubled as the time of hydrolysis was increased from ten minutes to forty-five minutes.

*Obtained through the kindness of A. S. Cook of Ayerst, McKenna and Harrison, Montreal, Canada.

To minimize the possible variations given by single isolated specimens and to insure an adequate supply of urine for future work, an 18-liter sample of pregnancy urine was collected by pooling twenty-four hour urine specimens from patients seven months pregnant. This pooled urine was preserved with chloroform, the pH adjusted to 7.0, and the urine filtered and stored at 3 to 5° C. The experiments to be described represent a comparison of a number of techniques all carried out on aliquots of this stock urine.

Bio-assay, by a modified Marrian and Parker method recently described by Thayer, Doisy, Jr., and Doisy,[16] was conducted on all estrogenic fractions.

After preliminary assays had determined the proper dose level, the final assays of an experiment were run on a group of animals available on a single assay day. A minimum of twenty animals was used at each assay level and twenty animals served as controls. Although great care was taken in these bio-assays, it must be kept in mind that differences in results of 20 per cent probably are not significant.

The yield of estrogen obtained in each of the experiments indicated that although ether extracts of hydrolyzed urines were further separated into phenolic, nonphenolic, and acidic fractions, colorimetric analysis by a modified sulfanilic acid method (Van Bruggen[17]) and by Bachman's method[1] gave results that differed markedly from the bio-assay results. Although a few such chemical determinations gave good agreement with bio-assay figures, for the most part the colorimetric determinations gave values from two to eight times higher than bio-assays.

Preliminary studies indicated that the ten-minute hydrolysis with 15 volumes per cent did not give complete hydrolysis of combined forms. To test this possibility, several experiments were carried out.

Experiments 1 and 2.—Of the 18-liter pooled sample, 1,200 ml. were acidified to 15 volumes per cent with HCl and refluxed in an all-glass apparatus fitted with a siphon. This arrangement permitted the withdrawal of samples of the boiling urine at various time intervals. One hundred fifteen milliter samples were withdrawn, cooled under water, and saturated with NaCl to help prevent emulsion formation. The estrogens were extracted from the treated urine with four 50 ml. portions of purified ethyl ether. The combined ether extracts were washed with 9 per cent $NaHCO_3$, dilute HCl, and distilled water. The ether was removed by distillation and the residue taken up in 95 per cent ethanol. The zero hour sample was taken after acidification but before the system was heated and should thus represent free estrogens present. A similar experiment was carried out but differed from the first in that 125 mg. of 1-amino-2-naphthol-4-sulfonic acid per 100 ml. urine were added to the urine. In addition, N_2 was passed through the urine for a ten-minute period before heating was started as well as during the hydrolysis. Table I lists the data obtained from these two experiments.

Experiment 3.—In this experiment, the method of Marrian[2] and several modifications of his method were studied. Three 100 ml. aliquots of the pooled urine were adjusted to a pH of 1.0 with HCl. To one of these an additional

TABLE I. HYDROLYSIS OF POOLED PREGNANCY URINE WITH 15 VOLUMES PER CENT HCl (COLUMN A, ACTIVITY RECOVERED WITHOUT PROTECTION; COLUMN B, ACTIVITY RECOVERED WITH N_2 AND THE PROTECTIVE AGENT)

SAMPLE	TIME OF HYDROLYSIS (MIN.)	M.U. PER LITER	
		A	B
1	0	37,812	34,300
2	10	75,050	98,250
3	20	91,313	98,250
4	30	110,000	75,000
5	40	96,250	80,000
6	50	110,000	98,000
7	60	89,375	121,000
8	90	75,625	60,500
9	120	86,400	70,000
10	240	------	50,000

1 ml. of concentrated HCl was added; to the other two, 3 ml. additional HCl were added. One of these two also received a small amount of the protective agent. Two other 100 ml. samples were adjusted to a pH of 1.00 with H_2SO_4. One of these received an additional 1 ml. of concentrated H_2SO_4; the other, 3 ml. of H_2SO_4. The five flasks were then simultaneously autoclaved at 120° C. for two hours. After this pressure hydrolysis, the samples were cooled and extracted by the technique mentioned in Experiment 1. Table II compares the results of the experiment on the five samples.

TABLE II. TWO-HOUR PRESSURE HYDROLYSIS AT 120° C. OF ALIQUOTS OF THE POOLED URINE, ALL SAMPLES BEING FIRST ADJUSTED TO pH 1.0 WITH THE APPROPRIATE ACID

SAMPLE	ADDITIONAL TREATMENT	M.U. PER LITER
1	1 ml. HCl per 100 ml. urine	116,880
2	3 ml. HCl per 100 ml. urine	123,750
3	3 ml. HCl per 100 ml. urine plus protective agent	132,000
4	1 ml. H_2SO_4 per 100 ml. urine	86,663
5	3 ml. H_2SO_4 per 100 ml. urine	103,120

Experiment 4.—The high activity of fractions 4-A and 7-B of Experiments 1 and 2 (Table I) after the ten-minute time as well as the good yield of the third fraction (Table III) suggested that the ten-minute hydrolysis with 15 volumes per cent HCl was not liberating all of the combined estrogens. This experiment was devised to determine the degree of hydrolysis effected by the Smith[12] method as performed in our experiments. One liter of the pooled urine was made acid to 15 volumes per cent with HCl and heated in the same apparatus used in Experiments 1 and 2. Samples were taken and extracted four

TABLE III. PRELIMINARY HYDROLYSIS AND EXTRACTION (COLUMN A) AND SUBSEQUENT RECOVERY OF ESTROGENS BY HYDROLYSIS AND EXTRACTION (COLUMN B)

SAMPLE	TIME OF HYDROLYSIS OF FIRST SAMPLE (A)	M.U. PER LITER		TOTAL RECOVERY (A PLUS B)
		A	B	
1	0	38,800	55,000	93,000
2	10	95,000	31,250	126,250
3	30	82,500	6,250	88,750
4	60	110,000	800	110,800
5	90	82,500	1,000	83,500
6	120	89,375	1,200	90,575

times with ether as before. However, after free estrogens had been extracted with ether, the urine residue was thoroughly extracted with butyl alcohol to remove conjugated forms. The butyl alcohol was concentrated, the residue dissolved in 100 ml. water, HCl was added to 15 volumes per cent, and the extract hydrolyzed for ten minutes; the estrogens were removed with ether as before. Each extract was assayed separately. In Table III, column A represents the activity obtained by the first hydrolysis and column B represents activity present in the hydrolyzate of the butanol extractions. Activity in B, then, represents estrogens not hydrolyzed or extracted by a simple acid hydrolysis.

Experiment 5.—The high activity of the B fraction of the ten-minute hydrolysis of the previous experiment might be due to one or both of two factors. Although the distribution coefficient of an acid ether partition strongly favors the movement of estrogens into the organic phase, it is possible that faulty extraction of the urine might account for the activity of the B fraction. On the other hand, incomplete hydrolysis at ten minutes might cause the retention of activity in the water phase. The following data were obtained in an effort to clear up this matter. An aliquot of the pooled urine was hydrolyzed for ten minutes with 15 volumes per cent HCl. After the customary ether extraction, the urine residue was extracted with butanol. The butanol extract was divided into two equal parts, one-half of which was concentrated at a low temperature under suction to avoid hydrolysis during this time. The other half of the butanol extract was concentrated and rehydrolyzed as in Experiment 3. This fraction then contains the ether-soluble estrogens after hydrolysis of the butanol extract. In the original ether extract 103,100 M.U. per liter were found; 6,000 M.U. per liter were found in the butanol extract that was not rehydrolyzed. The butanol fraction that was rehydrolyzed showed 15,000 M.U. per liter.

The effectiveness of continuous extraction and hydrolysis was studied in a number of experiments. It was felt that if the free estrogens could be extracted by an organic solvent immediately after their release from the conjugate form, that destruction might be minimized. Attempts to accomplish this by a number of methods are reported below.

Experiment 6.—One hundred milliliter samples of the pooled urine were made to 15 volumes per cent HCl and were then layered with 100 ml. amounts of toluene, butanol, benzene, and cyclohexanol, respectively. Such systems were boiled under reflux for two hours. During the heating period, good contact between the urine and the organic solvent was assured by the bubbling of a stream of nitrogen through the solutions. After the two-hour heating, the urine and solvent were cooled, the organic solvent layer removed, and the urine residue further extracted with three additional portions of the same solvent used during the heating period. Toluene gave a recovery of 41,300 M.U. per liter; butanol, 82,500; benzene, 64,800; and cyclohexanol, 70,000.

Experiment 7.—Since the original hydrolysis and extraction described by Dingemanse and co-workers[4] was for a considerably longer period of time, and used, in addition, a greater total amount of solvent, the procedure of Experiment 6 was not comparable. To approximate the conditions used by Dinge-

manse, 100 ml. of urine were acidified with 23 ml. HCl, the urine covered with 100 ml. benzene, and the mixture refluxed for four hours. The benzene was removed and replaced with 100 ml. fresh solvent and the urine and benzene again refluxed. A third such treatment gave a total of 300 ml. benzene used during a total hydrolysis and extraction period of twelve hours. The benzene solutions were combined, washed with a small amount of water, and taken to dryness. By this method, a total of 500,000 M.U. per liter was obtained.

Experiment 8.—The principle of the previously mentioned continuous hydrolysis and extraction procedure of Talbot and associates[15] was applied to our problem of estrogen recovery. The apparatus used was similar in construction to that described by Smith and Smith.[12] The lighter-than-water solvents used were delivered to the bottom of the urine phase by a tube having a porous glass disk at the bottom. The column of urine was surrounded, in addition, by a water bath that permitted the maintenance of the urine at elevated temperatures. With benzene as the circulating solvent, the urine was kept at 70° C. and 77,000 M.U. per liter were obtained; with toluene the temperature was 80° C., 77,000 M.U. per liter being found, and cyclohexanol permitted a temperature of 90° C. with a recovery of 100,000 M.U. per liter.

TABLE IV. HYDROLYSIS OF CONJUGATED ESTROGEN IN BUTYL ALCOHOL

SAMPLE	TIME OF HEATING	M.U. PER LITER
1	0	74,250
2	10	107,250
3	60	103,125
4	120	148,500

Experiment 9.—Liquid ammonia has within recent years been extensively employed in organic hydrolysis procedures. Hydrolysis with liquid ammonia (ammonolysis) was attempted in two ways. In Experiment 9a the total estrogens were extracted from urine with butanol. After the butanol was evaporated, the residue was taken up in 20 ml. dry butanol and 40 ml. liquid ammonia were added. The mixture (in an open glass tube) was enclosed in a bomb made of two-inch steel pipe capped at each end by ordinary pipe caps. After forty-eight hours at room temperature the residue was distilled to dryness and taken up in 95 per cent ethanol as usual. In Experiment 9b, a butanol extract of urine was taken to dryness, the residue transferred to the glass inner tube of the bomb, and the dry residue treated with liquid ammonia. A total of 48,125 M.U. per liter was obtained by the first treatment and 68,750 M.U. were obtained by the second method.

Experiment 10.—It was thought that if the total estrogens could be removed from the urine and the combined forms then hydrolyzed, that destruction might be reduced. To this end, 500 ml. of the pooled urine were made acid to congo red and extracted with five 100 ml. portions of butyl alcohol. The combined butanol extracts were washed once with water, made to 15 volumes per cent with HCl and hydrolyzed as in Experiment 1. One hundred thirty milliliter samples were withdrawn (equivalent to 100 ml. urine), cooled,

and concentrated alkali added until the aqueous phase which separated was just acid to litmus. The alcohol was concentrated and the residue taken up in ethanol.

Table IV gives the results of the previously described experiments in terms of mouse units per liter of urine.

DISCUSSION

In discussing the results of these experiments it should be kept in mind that the yields by the various techniques have been obtained by bio-assay and that differences of 20 per cent are not significant. Experiment 1 bears out the previous suggestion that, at least with the pooled urine used in this work, a ten-minute hydrolysis with 15 volumes per cent HCl does not hydrolyze all the conjugated forms present. Since boiling for periods greater than twenty to thirty minutes in 1.6 N HCl is known to destroy estrogens, it seems likely that the high activity present at forty minutes is due to the presence of a hydrolytic process and its predominance over a destructive process. There seems to be little advantage to using nitrogen and the sulfonic acid as in Experiment 2, although experiments on pure estrogens had demonstrated the effective protection of such treatment.

The data of Experiment 3 indicate that pressure hydrolysis is an effective method for urine hydrolysis. Protection of labile forms by the mild reducing agent was of definite value in this procedure. The good recovery of activity by the pressure hydrolysis method recommends it for a routine procedure.

Experiment 4 bears out the previous contention that in this work the ten-minute hydrolysis with 15 volumes per cent HCl does not give a quantitative yield of estrogens. The total of 93,000 units of the zero hour sample closely approximates the 95,000 units given by the regular ten-minute hydrolysis of the ten-minute sample. The 31,250 units of the rehydrolyzed specimen of the ten-minute sample, however, represent approximately a 25 per cent potential loss when the routine Smith[12] method is used. The activity of the rehydrolyzed specimens even up to 120 minutes might indicate the presence of some difficulty hydrolyzable or extractable form in pregnancy urine.

That the usual four extractions with ether may not remove all the supposedly ether-soluble forms is indicated by Experiment 5. The 6,000 units not extracted by the ether represent 5 per cent of the total activity potentially lost; while the sum of the second and third fractions (21,000 units) represents a 17 per cent over-all loss.

The protection offered by the layering used in Experiment 6 does not appear to have been outstanding. It is possible that better yields might have been obtained if shorter periods of heating had been used. In these four samples, the good yield by the use of butanol is in agreement with the results obtained in Experiment 10.

The low recovery of activity seen in Experiment 7 is probably due to the excessively long heating period used. The acid concentration used was somewhat greater than that recommended by Dingemanse[4] so that the method is not exactly as described by that author.

The use of continuous hydrolysis and extraction as in Experiment 8 seems to be of promise; however, the high boiling point (161.5° C.) of the cyclohexanol makes it undesirable as a routine solvent. Somewhat different periods of heating and temperature conditions might greatly improve this procedure.

Liquid ammonia as used in Experiment 9 did not cause appreciable hydrolysis of conjugated forms. A more refined bomb container which would allow the retention of higher pressure might greatly enhance the utility of this method.

The use of butanol as the solvent and hydrolyzing medium, as in the final experiment, appears to be of definite promise. Woolf, Viergiver, and Allen[18] have shown that the method of extraction used in this experiment would quantitatively extract all the pregnandiol glucuronide so that it is probable that most of the estriol complex was removed. The 74,000 units of the zero hour sample probably represent some hydrolysis of conjugated forms during the removal of the butyl alcohol from the sample. The 148,000 units present in the two-hour fraction were the highest yield obtained in these studies.

SUMMARY

A number of factors affecting the hydrolysis and extraction of urinary estrogens have been investigated. The use of 15 volumes per cent HCl for ten minutes as the hydrolyzing medium, although giving reasonably good results, does not appear to give optimum yields. Pressure hydrolysis under conditions similar to those advocated by Cohen and Marrian[2] is of definite advantage. The hydrolysis of combined forms in an organic solvent (butanol) gave the highest yields obtained. Preliminary work indicated that there might be wide differences in individual samples of urine; the use of a pooled sample presumably nullified these individual differences. It is possible that if studies such as have been presented in this paper were complemented by a fractional analysis of all urine samples, considerable light could be thrown on the hydrolysis and destructive reactions.

The author gratefully acknowledges the help of the staff of the Department of Biochemistry and especially the services of Miss Corinne Dewes.

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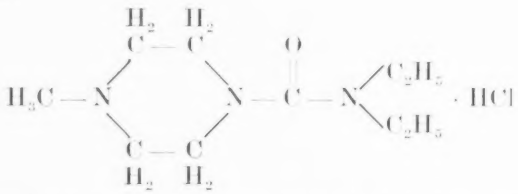
STUDIES ON THE CHEMOTHERAPY OF FILARIASIS

VI. Some Pharmacodynamic Properties of 1-Diethylcarbamyl-4-Methylpiperazine Hydrochloride, Hetrazan

B. K. Harned, Raymond W. Cunningham, Sybella Halliday, R. E. Vessey, N. N. Yuda, Mary C. Clark, Carolyn H. Hine, Rachel Cosgrove, and Y. SubbaRow
Pearl River, N. Y.

THE introduction by Hewitt and co-workers[1, 2] of 1-diethylcarbamyl-4-methylpiperazine hydrochloride as a filaricide effective in experimental animals and in man has necessitated an investigation of the pharmacologic properties of this compound. The use of piperazines in medicine is not new. For more than a decade prior to 1918[3] piperazine and 3,6-dimethylpiperazine were accepted remedies for the treatment of gout and rheumatism. Although these drugs were worthless in these diseases,[3, 4] the years of use served to establish the low toxicity of the nucleus and to provide excellent descriptions of the symptoms of overdosage.

1-Diethylcarbamyl-4-methylpiperazine hydrochloride, also referred to as Hetrazan and 84-L, has a molecular weight of 234.6[5] and has the following structural formula:



It is a colorless crystalline solid, highly soluble in water, alcohol, and chloroform, but insoluble in benzene, ether, and petroleum ether. The pH of a 1.0 per cent solution is 4.1. In the pharmacologic experiments the solutions were adjusted to pH 7.4.

METHODS

The acute toxicity of single doses was studied in six species. Multiple doses at short intervals were given to mice, rats, and dogs. Studies on the chronic toxicity were made with rats, rabbits, dogs, and chickens.

Tests for irritation were made by intracutaneous injections in guinea pigs of 0.1 c.c. of 1.0 per cent solutions and by local application of the same concentration to the eyes of cats. Antihistaminic action was tested on the isolated guinea pig gut and on guinea pigs in a spray-chamber. Cats were used for pupillary studies. Hemoglobin was determined as cyanmethemoglobin.[6] The Lipschitz assay[7] was used to evaluate diuretic action. Analgesia was determined by a method which employed the application of heat to the rat's foot.[8]

From Lederle Laboratories Division, American Cyanamid Co.
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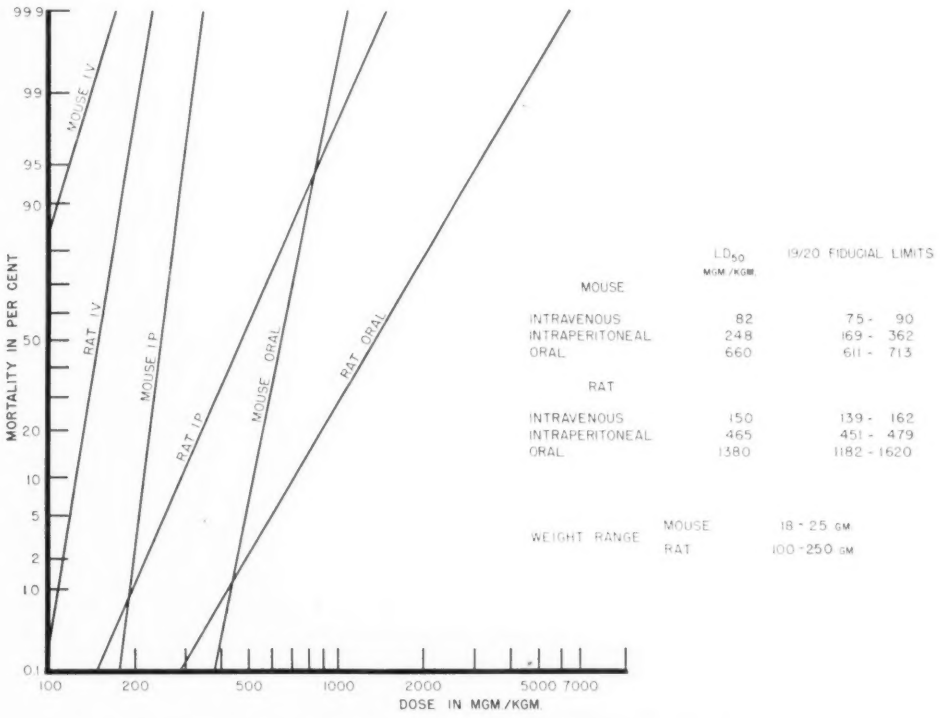
Respiratory movements were relayed to a kymograph by a string attached halfway between the inferior end of the sternum and the umbilicus. Electrocardiographic studies were made with a Cardiotron on anesthetized and unanesthetized dogs. In anesthetized animals blood pressure was recorded from the carotid artery. In unanesthetized dogs the pressures were recorded by femoral arterial puncture.

RESULTS

Acute Toxicity.—

Single Doses.—

Mice and Rats: The dose-mortality curves recorded in Fig. 1 summarize the data from 238 mice and 260 rats. Toxic doses produced convulsions that were predominately tonic. The convulsant dose, however, was considerably below the fatal dose.



ACUTE TOXICITY OF 1-DIETHYL CARBAMYL-4-METHYL PIPERAZINE HYDROCHLORIDE (84L)

Fig. 1.—The experimental results were plotted on log probability paper[9] and straight lines were fitted by eye. The 19/20 fiducial zones were estimated by a modification of the method of Litchfield and Fertig.[10] The modification allowed for the fact that the population was not homogeneous in all cases and consisted of correcting the estimated values by multiplying by $\sqrt{\frac{(\text{Chi})^2}{n}}$, as described by Wilcoxon and McCallan.[9]

Guinea Pigs, Rabbits, Cats, and Dogs: Lethal doses were not determined in these species but large doses were tolerated with few signs of toxicity. Ten guinea pigs given 50 mg. per kilogram intraperitoneally showed no changes. Objectionable reactions were not observed in fifteen rabbits given 100 mg. per kilogram by the same route. Twenty-five milligrams per kilogram given intra-

peritoneally to six cats and 50 mg. per kilogram given to three cats caused vomiting in six to ten minutes. In addition, these animals showed slight drowsiness.

Serious reactions were not observed in uanesthetized dogs that had received 100 mg. per kilogram orally or intraperitoneally or one-fifth of this dose by rapid intravenous injection. If reactions occurred they were, in order of frequency, nausea, vomiting, and muscular tremors. The tremors closely approximated those of a dog shivering from a low temperature. In addition to these reactions, intravenous injections caused stimulation of the respiration which lasted from one to three minutes (Fig. 3). Oral doses of 50 mg. or more per kilogram often produced emesis, but the presence of food in the stomach decreased the frequency of this occurrence. Oral doses of 25 mg. per kilogram administered with food usually were retained.[1] Table I contains the data on thirty-nine dogs.

TABLE I. REACTIONS OF UNANESTHETIZED DOGS TO HETRAZAN (84-L)

NUMBER OF DOGS	DOSE (MG./KG.)	ROUTE OF ADMINISTRATION	SYMPTOMS			
			NAUSEA	EMESIS	RESPIRATORY STIMULATION	SHIVERING
			INCIDENCE IN PER CENT			
11	5	Intravenous†	9	9	100	0
2	10	Intravenous†	0	0	100	0
4	20	Intravenous†	75	50	100	0
1	25	Intravenous†	100	0	100	100
1	50	Intravenous†	100	100	100	100§
2*	100	Intraperitoneal	100	100		100
5	50	Oral‡	80	60		20
12	100	Oral‡	66	66		8
1	200	Oral‡	100			100

Respiratory stimulation lasted about one minute; shivering lasted from thirty to sixty minutes; vomiting never was accompanied by continued retching or signs of malaise; recovery was excellent in all dogs.

*These dogs were given 100 mg. per kilogram twice daily for two days.

†The intravenous injections were completed within one minute.

‡Dogs were fed two to five hours before dosing.

§Severe muscular tremors.

Repeated Doses.—

Since the antifilarial data of Hewitt and associates[1] indicated the necessity for frequent administration of Hetrazan it seemed desirable to study the rate of its destruction or excretion. For this study experiments were designed to give rats and mice repeated injections at a rate which barely exceeded the capacity of the animal to eliminate the compound. The criteria of accumulation were the incidence of convulsions and the percentage of mortality.

In rats the intraperitoneal LD_{50} is 465 mg. per kilogram (Fig. 1); therefore, in the tolerance studies a single intraperitoneal dose of 300 mg. per kilogram was given at zero time, with additional doses of 100 mg. per kilogram at hours 2, 3, 4, 5, 6, 7, and 8. The incidence of convulsions provided a delicate indicator of the rate of elimination of the compound (Table II). At 300 mg. per kilogram 100 per cent of the animals convulsed, but after an interval of two hours an additional dose of 100 mg. per kilogram produced no convulsions.

The incidence of convulsions after the third and fourth doses was 9 per cent in each case and after the eighth dose, 45 per cent. Although the total dose given to these rats amounted to 1,000 mg. per kilogram, no increment subsequent to the initial dose produced convulsions in all rats. In three groups of rats, sixty-five animals, 300 mg. per kilogram never failed to produce convulsions. Thus it appears that under the foregoing conditions the rat is capable of eliminating approximately 100 mg. per kilogram per hour.

TABLE II. THE EFFECTS ON RATS OF MULTIPLE INTRAPERITONEAL DOSES OF 84-L REPEATED AT SHORT INTERVALS

INTERVAL SUBSEQUENT TO INITIAL DOSE (hours)	NUMBER OF RATS INJECTED	DOSE		INCIDENCE OF CONVULSIONS (%)	MORTALITY (%)
		SINGLE (MG./KG.)	CUMULATIVE (MG./KG.)		
0	45*	300	Initial dose	100.	31.1
	Group I†				
2	22	100	400	0.	0.
3	22	100	500	9.1	4.7
4	21	100	600	9.5	0.
5	21	100	700	19.0	0.
6	21	100	800	4.7	0.
7	21	100	900	28.6	0.
8	11	100	1000	45.5	0.‡
	Group II				
4	9	300	600	100.	22.2
5	7	150	750	43.	0.
6	7	150	900	43.	0.
7	7	150	1050	71.4	42.8‡

Range of weight in grams, 200 to 250.
*The survivors were divided into two groups, I and II.
†Combined results of a group of 10 and a group of 12 rats dosed on different days.
‡Twenty-four hours later all animals were in good condition.

Obviously the validity of this calculation rests on the assumption that the rat does not become resistant to the convulsant action of the compound. This question has been answered by the data on Group II in Table II. In this series the original dose of 300 mg. per kilogram was repeated after four hours. The incidence of convulsions was again 100 per cent and the mortality, 22 per cent. When additional doses of 150 mg. per kilogram per hour were given on the fifth, sixth, and seventh hours, the incidence of convulsions rose from 43 per cent on the fifth hour to 71 per cent on the seventh. These data show that rats do not become resistant to the convulsant action of 84-L and also that 150 mg. per kilogram per hour exceed the ability of the rat to eliminate the compound.

In Group I, Table II, the mortality from the initial dose of 300 mg. per kilogram was 31 per cent, and from the succeeding doses, 700 mg. per kilogram in six hours, 5 per cent. A similar experiment on mice has yielded data of the same order. Thus the administration of four times the LD_{99} over a period of eight hours produced a total mortality of 27 per cent (Table III). The rate of elimination in milligrams per kilogram per hour appeared to be greater in mice than in rats.

TABLE III. THE EFFECTS ON MICE OF MULTIPLE INTRAPERITONEAL DOSES OF 84-L REPEATED AT SHORT INTERVALS

INTERVAL SUBSEQUENT TO INITIAL DOSE (HOURS)	NUMBER OF MICE INJECTED	DOSE		MORTALITY	
		SINGLE (MG./KG.)	CUMULATIVE (MG./KG.)	PER DOSE (%)	CUMULATIVE (%)
0	30	200	Initial dose	0.	0.
1	30	100	300	0.	0.
2	30	150	450	6.7	6.7
3	28	150	600	0.	6.7
4	28	150	750	7.1	13.3
5	26	150	900	3.8	16.7
6	25	150	1050	8.0	23.3
7	23	150	1200	0.	23.3
8	23	150	1350	4.3	26.7
24	--	--	1350	59.1†	70.0†
72	--	--	1350	0.	70.0

*Twenty minutes after dose.

†Since death from 84-L usually occurs within thirty minutes after the injection, one hesitates to attribute this mortality to the pharmacologic action of the compound. However, these figures do not affect the conclusions, since 310 mg. per kilogram in a single dose produced a mortality of 99 per cent.

Etherized dogs tolerated 60 to 70 mg. per kilogram of 84-L given intravenously during a period of one hour. Dog 548 readily tolerated nine doses, each dose 5 mg. per kilogram, during a period of ninety minutes (Fig. 4) and Dogs 546 and 547 each received seventeen such doses in eighty minutes without endangering the respiration. Dog 549 (Fig. 4) tolerated seven doses of 10 mg. per kilogram during a period of sixty minutes but developed respiratory failure when the eighth dose was given on the seventieth minute. This dog was maintained without difficulty on artificial respiration. Dog 540 received four doses of 20 mg. per kilogram during forty minutes. The fifth dose, which brought the total to 100 mg. per kilogram in fifty-two minutes, produced respiratory failure. Artificial respiration maintained this animal in a satisfactory condition and twenty minutes after the fifth dose another injection of 20 mg. per kilogram was made without producing circulatory failure.

Unanesthetized dogs have been given 100 mg. per kilogram intraperitoneally twice daily for two days without producing signs of toxicity more severe than vomiting and mild muscular tremors (Table I). One hour after the injections the animals appeared normal.

Chronic Toxicity.—

Rats.—The intraperitoneal injection of 100 mg. of 84-L per kilogram five days per week for fourteen weeks did not affect the rate of growth or produce any unfavorable reactions in male rats (Fig. 2). The control and dosed groups each started with twenty animals. After fourteen weeks there were fifteen in the control and fourteen in the dosed group. At the end of the series of doses the mean hematologic findings on ten rats from each group were: (1) hemoglobin, grams per 100 c.c.: control, 13.1, dosed, 13.5; (2) red blood cells, millions per cubic millimeter: control, 8.3, dosed, 8.7; (3) white blood cells, thousands per cubic millimeter: control, 14.4, dosed, 19.3; (4) lymphocytes, per

cent: control, 61, dosed, 79; (5) neutrophils, per cent: control, 34, dosed, 19; (6) eosinophils, per cent: control, 3.7, dosed, 1.0. The pathologist* reported that no differences between the groups were found on examination of the tissues of the animals.

Rabbits.—Fifteen rabbits were given, intraperitoneally, 50 mg. per kilogram of the drug for fourteen weeks. The growth of the group was not significantly modified (Fig. 2). During the period, four animals died in the control

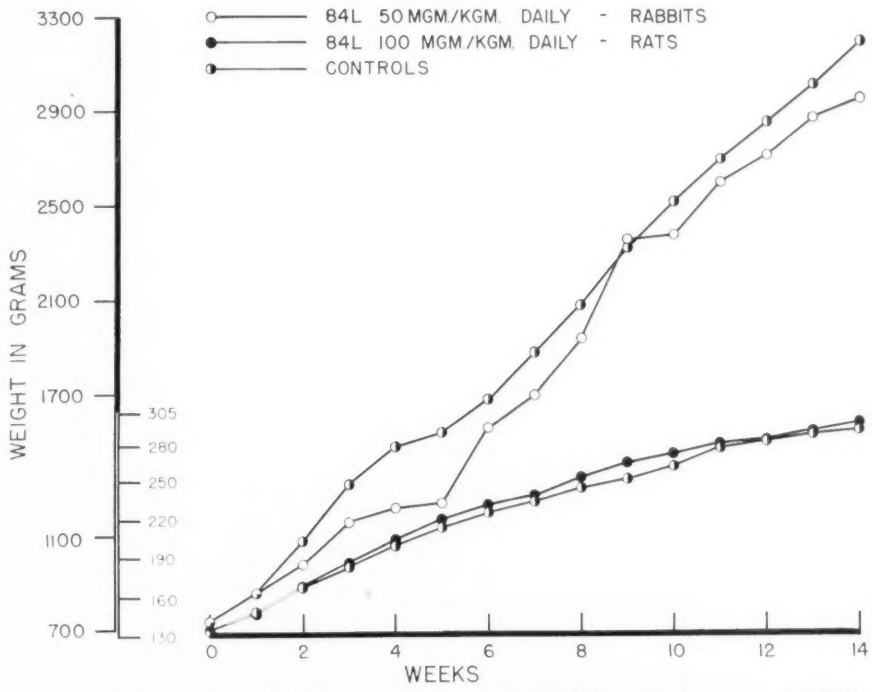


Fig. 2.

NUMBER OF ANIMALS	START	END
Control rabbits	15	11
Dosed rabbits	15	8
Control rats	20	15
Dosed rats	20	14

group and seven in the dosed, but the distribution of deaths does not suggest that they were due to chronic effects of the drug. After sixty-six doses during ninety-eight days the hematologic data revealed no difference between the control and the dosed groups. The average results for the groups were: (1) hemoglobin, grams per 100 c.c.: control, 11.1, dosed, 11.0; (2) red blood cells, millions per cubic millimeter: control, 6.1, dosed, 5.8; (3) white blood cells, thousands per cubic millimeter: control, 8.6, dosed, 10.3; (4) lymphocytes, per cent: control, 69, dosed, 70; (5) neutrophils, per cent: control, 28, dosed, 26.

*Dr. F. I. Dessau, Lederle Laboratories Division, American Cyanamid Co., made the examinations.

Dogs.—In a large series of dogs, Hewitt and associates[1] observed no evidence of chronic toxicity. Five of the dogs were given 50 mg. per kilogram intraperitoneally twice a day for thirteen days and two were dosed orally with 25 mg. per kilogram three times a day for sixty-four days. At the end of the period of dosing they were sacrificed and examined for pathologic changes,* but none were found that could be attributed to the treatment given.

Chicks.—Robbins' observation[11] that 2,4-dinitrophenol produced cataracts in the eyes of chicks prompted us to subject 84-L to a similar test. A group of eighteen 8-day-old White Rock chicks was divided into three balanced groups. Group 1 received a diet which contained 0.25 per cent 84-L and Group 2, 0.25 per cent 2,4-dinitrophenol; Group 3 was given the basal diet. Chicks on 2,4-dinitrophenol developed cataracts during the first twenty-four hours. The chicks in Group 1 were continued on the 84-L diet for fifteen days and there was never any evidence of lenticular changes. At the end of the experiment the lenses were sectioned and examined by a pathologist;† it was reported that the lenses were normal. The chicks fed 84-L grew at the same rate as the control animals in Group 3, and in appearance the individuals of the two groups were indistinguishable.

Miscellaneous Observations.—

General Behavior: Intraperitoneal doses of 50 mg. per kilogram given to dogs, cats, rats, and rabbits produced few signs. The dogs and rats were more sensitive to loud noises, but thirty minutes after the injections many of the animals in all the species tested appeared to be more quiet than usual; however, they were not asleep and responded normally to external stimuli.

Various tests disclosed that neither local anesthesia nor irritation were produced by 84-L.

Isolated Intestine: The activity on isolated rabbit ileum is of a low order. Concentrations of 1:100,000 in Tyrode's solution were required to give a perceptible relaxation of normal or spastic strips. The drug produced no effect on the guinea pig intestine.

Isolated Uterus: In concentrations of 1:100,000 84-L produced no effect on the isolated uterus from the rabbit or the rat. Virgin guinea pig uteri responded with weak contractions to a 1:100,000 concentration. In lower concentrations the response was barely perceptible or was absent.

The antihistaminic action on the guinea pig ileum was barely detectable and amounted to 1/2,000 to 1/10,000 of the activity of some of the clinically used compounds. Ten guinea pigs were injected intraperitoneally with 50 mg. per kilogram of 84-L and thirty minutes later were subjected to a standardized spray of histamine. Nine animals convulsed in three minutes and of these one died. One guinea pig withstood the spray for ten minutes with no signs other than dyspnea. A retest of this guinea pig one and one-half hours after 84-L produced convulsions in seven minutes. All of a group of ten control guinea

See footnote, page 221.

†Dr. E. Woll, Lederle Laboratories Division, American Cyanamid Co.

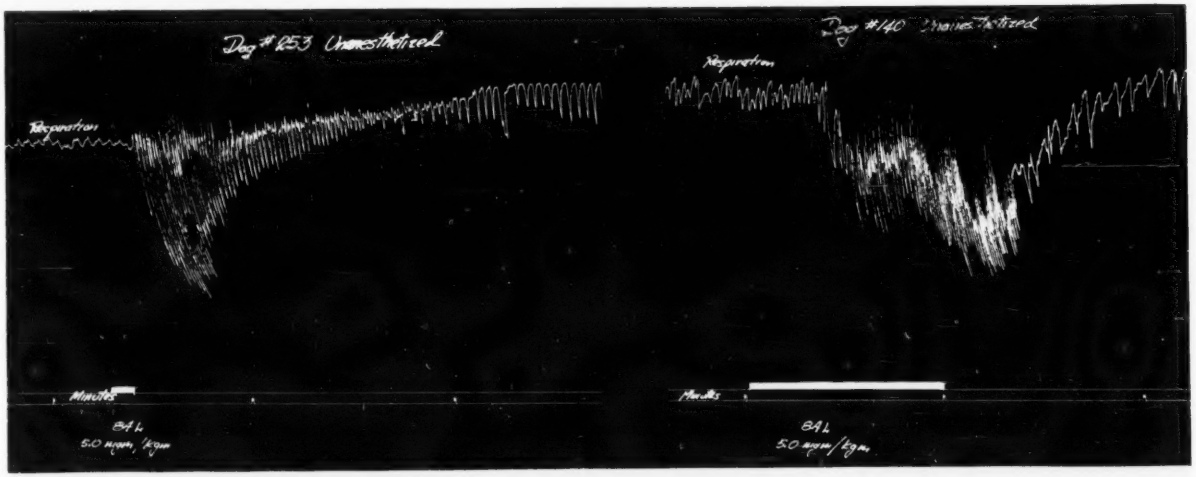


Fig. 3.—Typical records of respiratory movements in unanesthetized dogs after intravenous injection of 84-L. The signal line marks the duration of the injection. The respiratory movements were relayed by a thread attached halfway between the umbilicus and the end of the sternum.

pigs convulsed in three minutes and three died. From these data we conclude that 84-L does not exaggerate the action of histamine but exerts little protection against it.

Eye: Six cats were given the drug in a dosage of 25 mg. per kilogram intraperitoneally and three were given 50 mg. per kilogram. No evidence of myosis or mydriasis was observed. The local application of a 1.0 per cent solution produced no change in the pupil.

Blood Sugar: In subconvulsant doses 84-L had no effect upon the blood sugar.[12]

Diuretic Action: By the Lipschitz assay the diuretic potency is 17.5 times that of urea, or about one-half the effectiveness of caffeine.

Analgesia: In rats the compound produced evidence of a mild analgesia. The action was less than that produced by aminopyrine.

Respiration: Changes in respiration have not been observed except after intravenous doses. In unanesthetized dogs the intravenous injection of 5 mg. per kilogram in eight seconds stimulated the rate and depth of respiration for about a minute. Within two to three minutes from the start of the injection the respiration was normal (Fig. 3). When the same dose was injected over a period of sixty seconds, the stimulation was of a lower order and of a shorter duration. The injection of 20 mg. per kilogram in eighteen seconds produced an intense stimulation of respiration which lasted about a minute. In three minutes the animal appeared to be normal. In etherized dogs the respiratory changes were much less prominent.

Intravenous doses of 2 to 15 mg. per kilogram inhibited the respiration of normal and anesthetized rabbits. In unanesthetized rabbits the rapid injection of 5 mg. per kilogram produced an apnea lasting from five to ten seconds. Recovery was complete in three minutes. A dose of 2 mg. per kilogram injected in sixty seconds decreased the amplitude and slowed the respiration for thirty seconds. Twenty-five milligrams per kilogram injected in one minute killed one of two rabbits.

In anesthetized dogs lethal doses of 84-L produced death by respiratory failure. These animals could be maintained on artificial respiration long after breathing had ceased.

Circulation: In etherized animals the response of the blood pressure to effective doses of 84-L (Fig. 4, Table IV) resembled that produced by epinephrine. The principal difference noticed was a longer duration of the depressor phase. Doses of 0.1 and 0.5 mg. per kilogram were ineffective. Some dogs responded feebly to 1 mg. per kilogram, but 5 mg. per kilogram produced a quick, short-lasting rise in the blood pressure followed by a fall of somewhat longer duration. As the dose was increased the peak of the rise increased until it reached a maximum at about 20 mg. per kilogram. The changes in the blood pressure produced by initial doses ranging from 5 to 40 mg. per kilogram have been recorded in Table IV.

Fig. 4.—The effect of repeated doses of S4-L on the blood pressure of etherized dogs. In each series the signal line marks the time for the repetition of the original dose.

TABLE IV. THE EFFECT OF INITIAL INTRAVENOUS DOSES OF 84-L ON THE BLOOD PRESSURE OF ETHERIZED ANIMALS

ANIMAL	BLOOD PRESSURE CONTROL (MM. HG)	INTRAVENOUS* DOSE (MG./KG.)	BLOOD PRESSURE			
			RISE		FALL	
			MAXIMUM (MM. HG)	DURATION (MIN.)	MAXIMUM (MM. HG)	DURATION (MIN.)
Dog						
504	136	5	26	0.5	34	1.3
503	165	5	33	0.3	37	1.5
502	152	5	36	0.5	27	2.0
501	120	5	36	0.5	38	2.5
506	133	5	28	0.2	29	2.5
522	142	5	85	0.5	0	–
488	106	10	54	1.0	26	†
A-1	95	10	51	2.5	0	–
A-4	142	10	50	0.5	66	5.0
540	130	20	50	0.5	34	13 plus
A-2	120	20	92	1.2	40	9 plus
545	122	25	46	1.3	48	5 plus
489	112	25	28	1.0	52	†
490	115	25	39	2.0	48	†
491	145	25	91	4.0	0	–
544	156	25	38	1.0	50	5 plus
A-3	104	40	42	0.5	36	35
Cat						
498	136	10	12‡	1.5	0	–
492	120	23	40	2.0	0	–
Rabbit						
495	98	10	42	15.0	0	–
493	68	25	26	3.0	0	–
494	88	25	Fatal dose			

*The injection was completed in ten to thirty seconds.
†Fall was interrupted by the injection of another compound.
‡This rise was preceded by a fall of 8 mm. Hg. The duration of the fall was 0.3 minutes.

The leg plethysmograph readily demonstrated that the sharp rise in blood pressure produced by intravenous doses of 84-L in dogs was accompanied by an equally sharp decrease in the volume of the leg (Fig. 5). However, the vasodepressor phase is poorly reflected.

The close similarity between the response of the blood pressure to 84-L and to epinephrine, coupled with the observation that 84-L exaggerated the action of epinephrine, suggested that it inhibited either the cardiac vagus or those reactions which destroy epinephrine.

The vasodepressor response to stimulation of the right vagus before and after 84-L showed that the intravenous injection of 25 mg. per kilogram blocked 25 to 100 per cent of the vagal activity for a period of thirty minutes (Table V). The exaggeration of the pressor response of epinephrine parallels the inhibition of the vagus. Doses of 5 and 10 mg. per kilogram of 84-L produced essentially no vagal inhibition (Table V). The mechanism of inhibition is interesting since in doses which completely inhibited the cardiac vagus 84-L had no effect on the vasodepressor response to acetylcholine (Fig. 6).

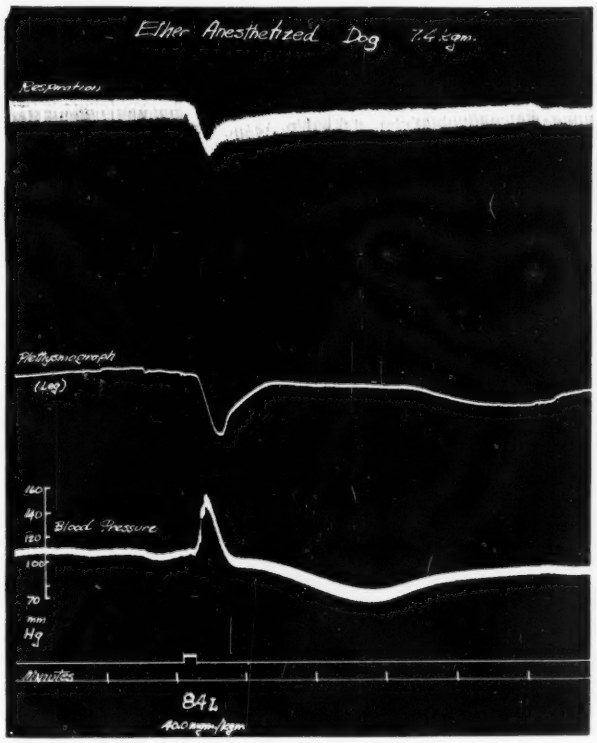


Fig. 5.—The effect of 84-L on the blood pressure, leg volume, and respiration of an etherized dog.

TABLE V. THE INHIBITION OF THE CARDIAC VAGUS BY 84-L

ANIMAL*	CONTROL VAGAL STIMUL. FALL IN BLOOD PRESSURE (MM. HG)	INTRAVENOUS DOSE (MG./KG.)	MINUTES AFTER INJECTION 3	15	30
			VAGAL INHIBITION (%)		
Cat					
498	22	10.0	45	18	0
498	12	15.0	0		
498	16	25.0	100	81	81
492	31	25.0	80	61	0
492	23	25.0	56	39	0
Rabbit					
493	26	25.0	100		19
493	20	12.5	0		
Dog					
544	76	25.0	100	100	68
489	64	25.0	100	100	46
545	66	25.0	100	48	42
490	70	25.0	85	78	69
487	56	25.0	100	100	100†
488	60	10.0	0		
488	60	25.0	75‡		
491	100	25.0	50	60	25
542	74	5.0	8	0	
542	73	10.0	7		

The right vagus was used; the blood pressure was recorded from the right carotid artery.

*All animals except Dog 491 were anesthetized with ether. In Dog 491 the anesthetic was intravenous sodium pentobarbital.

†Complete inhibition for at least two hours.

‡Vagal activity was normal at forty-five minutes.

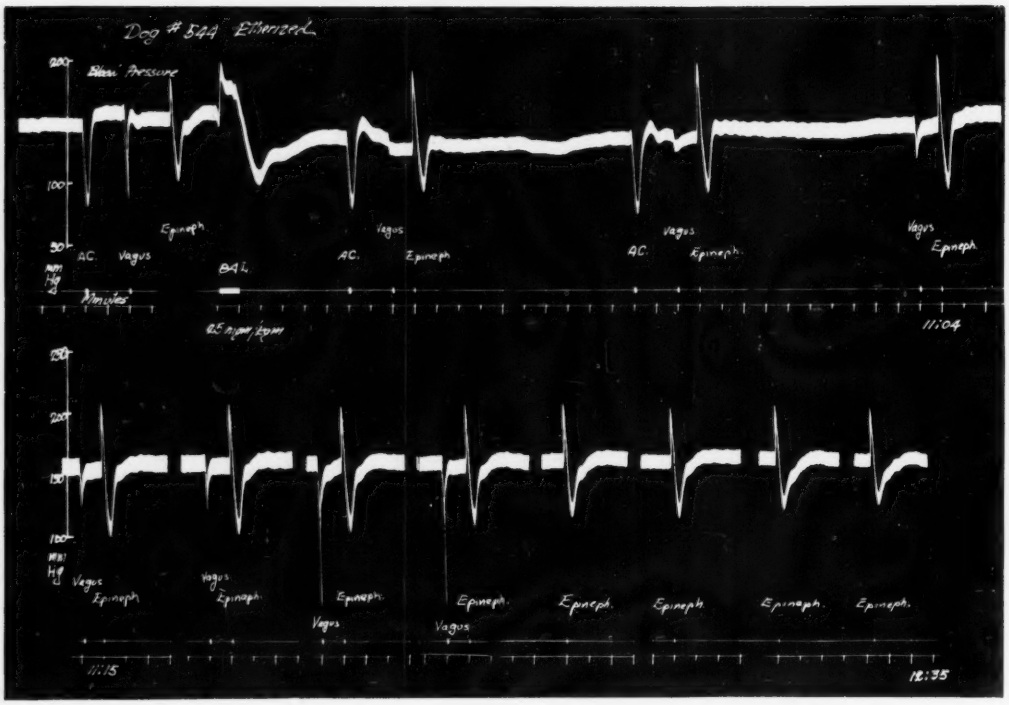


Fig. 6.—The action of 84-L on the response of the blood pressure to acetylcholine, epinephrine, and faradic stimulation of the right vagus. The doses per kilogram were: 84-L, 25 mg.; acetylcholine, 0.4 μg; and epinephrine hydrochloride, 2 micrograms.

Repeated injections produced a gradual diminution in the responses of the blood pressure and finally a complete disappearance of all action (Fig. 4). These data may provide a key to the explanation of the disappearance of the headache which developed in some patients after a few doses.[2]

Dogs with cords sectioned at approximately the second cervical vertebra and with all superior connections completely removed showed unmistakably that the vasopressor response of 84-L was not dependent upon centers in the brain. After this operation the vasopressor action of 84-L was exaggerated[12] (Fig. 7). Rises of blood pressure of 100 to 180 mm. Hg were common. Furthermore, the duration of the rise was much longer than in the etherized dogs. After the rise there was no fall unless the compound had been given before the blood pressure reached a stable level.

Electrocardiograms* obtained from etherized dogs which had been injected intravenously with 5 to 50 mg. of 84-L per kilogram revealed minimal changes. The heart rate was increased from 150 to 180 beats per minute during the hypertensive phase, but returned to the initial rate during the hypotensive phase. Regular sinus rhythm was maintained and such changes in the form of the complex as were noted were those usually associated with rapid heart

*The electrocardiographic studies were made by Dr. Maynard B. Chenoweth, Cornell University Medical College.

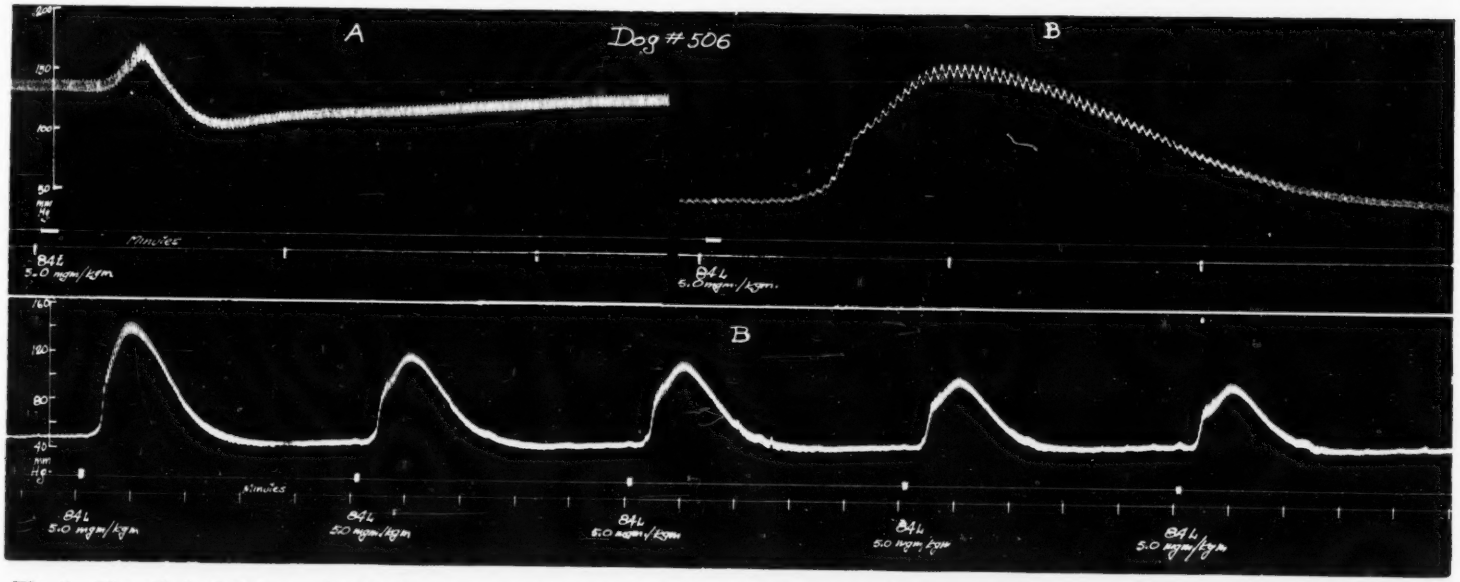


Fig. 7.—The effect of 84-L on the blood pressure before (*A*) and after (*B*) section of the cervical cord. Both vagi were sectioned before the control injection (*A*). The injections in B were made at five-minute intervals.

rates. Electrocardiograms taken during the terminal stages revealed no changes not typical of anoxia.

Cardiovascular responses in unanesthetized dogs: In five unanesthetized dogs the rapid intravenous injection (ten seconds) of 5 mg. of 84-L per kilogram produced an increase in blood pressure. Expressed in millimeters of mercury the elevations were: 68, 68, 68, 88, and 118.* When the same dose was given in sixty seconds the rise was 31 mm. Hg. Only two dogs were tested. These data indicated that the cardiovascular system of an unanesthetized dog was more sensitive to 84-L than that of the etherized animal. Electrocardiograms reflected similar differences. Although the rapid intravenous injection of 5 mg. per kilogram of 84-L produced insignificant changes in the electrocardiogram of etherized dogs, the same dose in unanesthetized dogs produced a number of changes in the rate and pacemaker location. Sinus arrest for periods as long as nine seconds and irregular rapid sinus rhythm interspersed with premature junctional and ventricular contractions were observed. Approximately five minutes after the injection the electrocardiogram was normal. Doses of 0.1 to 0.5 mg. per kilogram produced no changes or only slight changes in the rate and form of the electrocardiogram.

FATE OF COMPOUND.—

The absence of accumulative effects after multiple doses of 84-L (Tables II and III) suggested that the animals quickly became highly tolerant or that the compound was rapidly destroyed or excreted. Although a certain vascular tolerance to the drug developed, no comparable tolerance was seen in any other system.

Liver.—The role of the liver in the detoxication was examined by subjecting rats first to a series of doses of carbon tetrachloride and then, before hepatic recovery, to multiple doses of 84-L. The quantity of 84-L used slightly exceeded that which the rat was able to destroy or excrete so that any limitation in the ability of a key organ to perform its function would be reflected in a greater incidence of reactions in the test group. Rats weighing from 180 to 210 grams were given orally 0.166 c.c. of carbon tetrachloride per 100 grams of body weight. This dose was diluted to 0.5 c.c. with corn oil (Mazola) and given on days 1, 2, 3, 4, 5, 7, and 8. The tests for liver damage and 84-L catabolism were made on the first day after the last dose of carbon tetrachloride. The function of the liver was tested by following the duration of the anesthesia produced by sodium pentobarbital, a drug which is destroyed principally in the liver.[13] The results shown in Table VI demonstrate that a dose of 30 mg. per kilogram of sodium pentobarbital anesthetized 95 to 100 per cent of the control and the carbon tetrachloride-treated rats. Duration of the anesthesia as measured by the righting reflex was one hour in the control group and seven hours in the treated group (Table VI). The administration of 84-L in multiple graduated doses to rats treated similarly with carbon tetrachloride gave little evidence of an exaggeration of the effects seen in normal rats (Table VII). These data suggest that the liver is unimportant in the detoxication of 84-L.

*Coagulation of blood interfered with the evaluation of the depressor phase.

TABLE VI. THE EFFECT OF PREDOSING WITH CARBON TETRACHLORIDE ON THE DURATION OF ANESTHESIA PRODUCED BY SODIUM PENTOBARBITAL IN RATS; A TEST FOR LIVER FUNCTION

TIME AFTER SODIUM PENTOBARBITAL* (HOURS)	PERCENTAGE OF RATS WITHOUT RIGHTING REFLEX	
	TREATED† (7 DOSES CCL_4‡)	CONTROL†
0.5	95	100
0.7	95	70
1.0	95	5
2.0	95	0
3.0	95	0
4.0	100	0
5.0	100	0
6.0	100	0
7.0	94	0
7.0+	86	0
Overnight	23	0

*Thirty milligrams of sodium pentobarbital per kilogram, intraperitoneally.

†Twenty rats; weight, 140 to 170 grams at time of test.

‡The carbon tetrachloride was dissolved in corn oil (Mazola), 1 c.c. diluted to 3 c.c.; the dose, 0.5 c.c. of the mixture per 100 grams of body weight, was given orally on days 1, 2, 3, 4, 5, 7, and 8.

TABLE VII. THE MORTALITY PRODUCED BY 84-L GIVEN IN MULTIPLE DOSES TO RATS PRETREATED WITH CARBON TETRACHLORIDE*

GROUP†	INTERVAL SUBSEQUENT TO INITIAL DOSE (HOURS)	NUMBER OF RATS INJECTED	DOSE		MORTALITY	
			SINGLE (MG./KG.)	CUMULATIVE (MG./KG.)	PER DOSE (%)	CUMULATIVE (%)
Treated (7 doses of CCl_4)	0	29	300	Initial dose	10	10
	1	--	---	---		
	2	26	100	400	0	10
	3	26	100	500	0	10
	4	26	100	600	4	14
	5	25	100	700	4	17
	6	24	100	800	0	17
	7	24	100	900	12	27
Control	0	20	300	Initial dose	5	5
	1	--	---	---		
	2	19	100	400	0	5
	3	19	100	500	0	5
	4	19	100	600	0	5
	5	19	100	700	0	5
	6	19	100	800	0	5
	7	19	100	900	10	15

*The dose of carbon tetrachloride is given in footnote ‡ to Table VI.

†Twenty treated rats and twenty control rats; weight range in grams, 140 to 170.

Kidneys.—The administration of multiple doses of 84-L to unilaterally and bilaterally nephrectomized rats demonstrated the importance of the kidney in the elimination of this compound (Tables II and VIII). In sixty rats the left kidney was removed and after four or five days the animals were injected intraperitoneally with repeated doses of 84-L. The schedule of doses was 300 mg. per kilogram initially followed on hours 2, 3, 4, 5, 6, 7, and 8 by additional doses of 100 mg. per kilogram. Thus, in a period of eight hours the total cumulative dose was 1,000 mg. per kilogram. With this schedule the accumulated mortality for the eight hours was 80 per cent in the unilaterally nephrectomized rats and 34 per cent in the normal rats.

There were two groups of bilaterally nephrectomized rats (Table VIII). In one group the total nephrectomy was accomplished six days after the removal of the left kidney. The 84-L was administered twenty-four hours after the last operation. The initial dose was 200 mg. per kilogram with subsequent doses of 100 mg. per kilogram given on the second, third, fourth, and fifth hours. After an accumulated dose of 400 mg. per kilogram 50 per cent of the rats were dead, and after 600 mg. per kilogram the mortality was 100 per cent.

TABLE VIII. THE MORTALITY PRODUCED BY 84-L GIVEN IN MULTIPLE DOSES TO NEPHRECTOMIZED RATS

GROUP†	INTERVAL SUBSEQUENT TO INITIAL DOSE (HOURS)	NUMBER OF RATS INJECTED	DOSE		MORTALITY	
			SINGLE (MG./KG.)	CUMULATIVE (MG./KG.)	PER DOSE (%)	CUMULATIVE (%)
I	0	60	300	Initial dose	58	58
Unilateral	1	--	---	---		
nephrectomy	2	25	100	400	0	58
4-5 days be-	3	25	100	500	0	58
fore initial	4	25	100	600	16	65
dose	5	21	100	700	5	67
	6	20	100	800	15	72
	7	17	100	900	24	78
	8	13	100	1000	8	80
II	0	28	200	Initial dose	18	18
Left kidney	1	--	---	---		
removed 6	2	23	100	300	17	33
days before	3	19	100	400	26	50
initial dose;	4	14	100	500	71	86
right kidney	5	4	100	600	100	100
removed 24 hours before initial dose						
III	0	15	200	Initial dose	0	0
Total neph-	1	--	---	---		
rectomy	2	15	100	300	0	0
½-2 hours	3	15	100	400	7	7
before ini-	4	14	100	500	44	47
tial dose	5	8	100	600	75	87

*Effect of 84-L on normal rats is summarized in Table II, Group I.
†Weight range in grams: Groups I and II, 170 to 200; Group III, 204 to 240.

In normal rats the LD_{50} for a single dose was 465 mg. per kilogram (Fig. 1) and a single dose of 600 mg. per kilogram produced a mortality of 75 per cent. When the same schedule of doses was used on a group of rats which had been bilaterally nephrectomized under ether one-half to two hours before the first dose of 84-L, an accumulated dose of 500 mg. per kilogram killed eight of fifteen rats and 600 mg. per kilogram produced a mortality of 87 per cent. As an additional control on mortality attributable to the operation, the survival of thirteen totally nephrectomized rats was followed. Twenty-four hours after the operation the survival was 85 per cent and after forty-eight hours, 54 per cent. Attention should be called to the fact that the residual effects of ether in rats which were dosed on the same day that they were operated provided a significant protection against the lower doses of 84-L.

The value of the kidney in the elimination of 84-L has been shown graphically in Fig. 8. Here the mortality in per cent has been plotted on logarithmic probability paper for the cumulative doses administered to normal and to unilaterally and bilaterally nephrectomized rats, and these curves have been compared with the standard intraperitoneal dosage-mortality curve (Fig. 1). The mortality for the cumulative doses in the normal rats did not change significantly after the first dose. In the unilaterally nephrectomized rats the mortality from the initial dose was higher than in the unoperated animals and con-

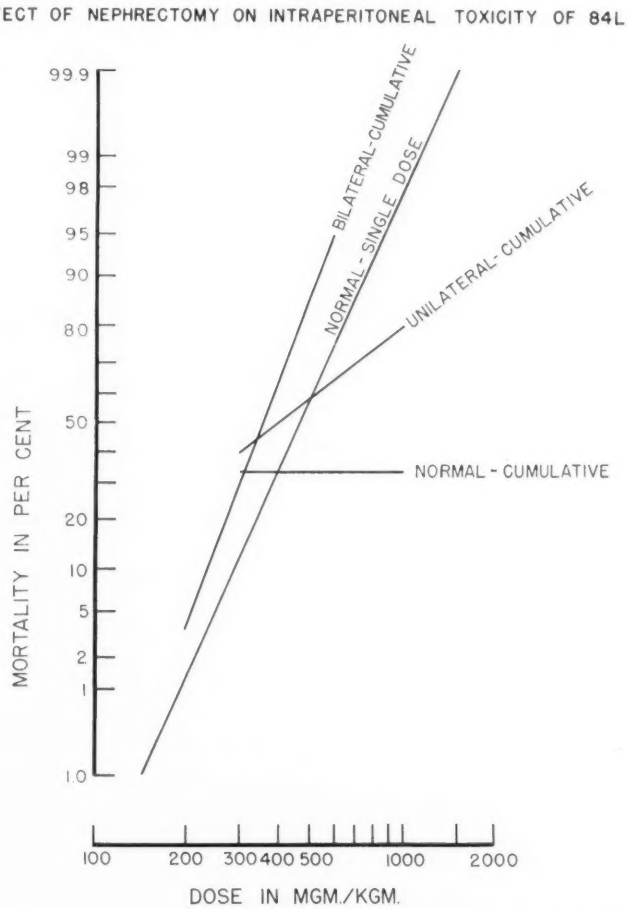


Fig. 8.—The schedule for the cumulative dosing of normal and unilaterally nephrectomized rats was: initial dose, 300 mg. per kilogram at zero time, and 100 mg. per kilogram at 2, 3, 4, 5, 6, 7, and 8 hours. For the bilaterally nephrectomized rats the schedule was: initial dose, 200 mg. per kilogram at zero time, and 100 mg. per kilogram at 2, 3, 4, and 5 hours.

tinued to mount as the doses accumulated. However, the mortality from these accumulated doses did not equal that produced by equivalent single doses. The dosage-mortality curve for the accumulated doses in the bilaterally nephrectomized rats paralleled and closely approximated that for the single doses to normal rats.

Excretion.—Although the data indicate that the elimination of the compound is accomplished principally by the kidney, the form in which it is excreted has not been determined. No practical chemical test for small quantities of the compound has been devised. However, a bio-assay on filaria-infested rats indicated that the equivalent of 63 per cent of a 300 mg. per kilogram intraperitoneal dose was excreted during the first twenty-three hours. In spite of the fact that nothing is known about the metabolic fate of 84-L, the low antifilarial activity of its relatives[14] strongly suggests that, in the rat, most of the compound is excreted unchanged.

SUMMARY AND CONCLUSIONS

1-Diethylcarbamyl-4-methylpiperazine hydrochloride, also known as Hetrazan and 84-L, has a low toxicity and causes few side reactions.

The intraperitoneal LD_{50} in mice was 248 mg. per kilogram and in rats, 465 mg. per kilogram. The oral LD_{50} in mice was 660 mg. per kilogram and in rats, 1,380 mg. per kilogram.

Mice, rats, rabbits, and dogs readily tolerated intraperitoneal injections of 100 mg. per kilogram.

Daily intraperitoneal doses of 50 mg. per kilogram in rabbits and 100 mg. per kilogram in rats, five days per week for fourteen weeks, produced no evidence of toxicity. Twenty-five milligrams per kilogram twice a day, orally, for two months produced no evidence of toxicity in dogs.

The compound was not irritating; it produced no local anesthesia and had no effect on the eye, on the isolated uterus or intestine, or on the blood sugar. It was mildly diuretic and analgesic. Intravenous doses of 2 to 25 mg. per kilogram in unanesthetized dogs stimulated the respiration. The heart and blood pressure were not affected by rapid intravenous injections of 0.5 mg. per kilogram, but larger intravenous doses in unanesthetized dogs produced a transient deviation from the normal.

The compound was rapidly excreted by the kidney. In rats and mice the rate per hour was approximately one-third of the intraperitoneal LD_{50}.

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LABORATORY METHODS

SCREENING METHOD FOR BLOOD GLUCOSE*

HUGH L. C. WILKERSON, M.D.,† AND ERICH HEFTMANN, PH.D.‡
BOSTON, MASS.

IN THE course of mass surveys carried out by this Section[1] it became increasingly evident that a considerable saving of time and money might be effected through elimination of definitely normal blood samples from routine glucose analysis by means of a simplified test. The method is based on that of Hagedorn and co-workers[2] but the steps requiring experience in chemical techniques and fluid reagents have been eliminated. The time required for a single analysis is about five minutes, the amount of blood, 0.1 milliliter. This paper describes the screening method and gives the results obtained with the first fifty blood samples.

REAGENTS AND APPARATUS

All reagents are in tablet form.§ In Boston (and presumably in many other localities) ordinary tap water may be used instead of distilled water.

Tablet 1

$ZnSO_4 \cdot 7H_2O$	10 mg.
NaCl	190 mg.
Talc	As binder
Mineral oil	As lubricant

Tablet 2

KI	100 mg.
$NaHCO_3$	10 mg.

Tablet 3

$K_3[Fe(CN)_6]$, recrystallized	1.28 mg.
Na_2CO_3, anhydrous	6.5 mg.
Starch, soluble	1 mg.
NaCl	92 mg.

Tablet 4

Tartaric acid	80 mg.
$ZnSO_4 \cdot 7H_2O$	20 mg.
Starch	As binder

Heating Tablets

Methenamine.‖

The apparatus consists of a Pyrex test tube 16 by 150 mm., graduated at 5 ml., the mouth of which is widened into a funnel, a scoop made of a piece of glass rod, and a portable test tube stand. The stand is so constructed that the

*United States patent applied for.

†Senior Surgeon, Diabetes Section, United States Public Health Service.

‡Biochemist, Diabetes Section, United States Public Health Service.

From the United States Public Health Service, Diabetes Section.

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§We gratefully acknowledge the generous help of the staff of Eli Lilly & Company Indianapolis, Ind., who prepared and furnished reagent tablets.

‖Reagent tablets No. 1588, Eli Lilly & Company, Indianapolis, Ind.

test tube can be heated in a reproducible manner by the heating tablets, that is, the distance between tube and tablets is constant (five-eighths of an inch) and drafts are kept out.

PROCEDURE

Fill the test tube with water to the 5 ml. mark. Obtain 0.1 ml. capillary blood from the fingertip or ear lobe by means of a capillary pipette. Deliver and rinse the blood into the water in the tube. Add one Tablet 1 and one Tablet 2 to the tube, put it on the stand, and light two heating tablets underneath it. The blood proteins are coagulated by the zinc hydroxide and form a scum which floats on top of the liquid. As the solution boils, the cake of protein is pushed upward in the test tube by the steam and can be removed with the glass scoop when it reaches the funnel-shaped mouth. The funnel prevents the solution from boiling over and unremoved protein from falling back into the tube. When both heating tablets are nearly consumed, light another heating tablet on them and add one Tablet 3 to the test tube. At the end of the second heating period cool the test tube by immersion in cold water and add Tablet 4. When cold, the solution will be either blue, due to the formation of the starch-iodine complex, or colorless, if no iodine is present.

RESULTS

The results of the screening method were checked against the blood sugar method of Somogyi[3, 4] as modified by Nelson.[5] In order to obtain a more accurate value for the true sugar concentration of the blood, the Somogyi-Nelson determination was carried out on a 1.0 ml. sample of venous blood, the 0.1 ml. sample for the screening method being taken from the same sample of venous blood. The samples were taken from normal and diabetic subjects.*

Fifty blood samples were subjected to the screening method, then analyzed by the Somogyi-Nelson method. Twenty-five samples, having a true sugar concentration of 170 mg. per cent or less by the Somogyi-Nelson method, gave a blue color (negative reaction) in the screening method. Four samples had blood sugar values between 170 and 180 mg. per cent and gave faint or dark-blue colors (negative) or no color (positive) reaction. None of the twenty-one blood samples containing more than 180 mg. per cent gave a color reaction in the screening test.

DISCUSSION

The amount of potassium ferricyanide in Tablet 3 is so adjusted that it will be completely reduced under the conditions of the test by the glucose present in 0.1 ml. blood when the concentration is 180 mg. per cent or more. If the glucose concentration in the blood sample is lower than 170 mg. per cent, some potassium ferricyanide is left over to oxidize the potassium iodide in Tablet 1 to iodine which produces a blue color with starch upon acidification by Tablet 4. The variability of the results between 170 and 180 mg. per cent is due to variation in the tablets, the amount of water, heating, and so forth.

*We wish to thank Dr. A. Marble of the New England Deaconess Hospital for making available the clinical material.

The 180 mg. per cent level for capillary glucose has been chosen arbitrarily as the value above which a retest by a conventional blood sugar method seems warranted. Naturally, this level may be adjusted at will by altering the conditions of the test, for example the potassium ferricyanide content of Tablet 3. Inasmuch as a knowledge of the actual blood sugar value is less important in screening diagnostic work than whether or not a certain level is exceeded, the test recommends itself wherever laboratory facilities are unavailable.

SUMMARY

A simple and inexpensive screening method for blood glucose has been described. A blood sample of 0.1 ml. may be classified as above or below a certain glucose concentration within five minutes by means of this test. In the first fifty cases examined by this method, twenty-five blood samples below 170 mg. per cent glucose gave a negative result, twenty-one samples above 180 mg. per cent a positive result, and four samples between 170 and 180 mg. per cent both positive and negative results.

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AN INCREASE OF COMPLEMENT UNITS BY THE USE OF EGG ALBUMIN

S. W. Bohls, M.D.,* and Phyllis Shaw, B.S., M.T.,† (ASCP)
San Antonio, Texas

THE behavior of complement and amboceptor titrations in complement fixation tests for the serodiagnosis of syphilis has been under study in Santa Rosa Hospital Laboratory for some time. Literature pertaining to the subject is meager.

Periodically difficulty has been encountered, particularly in the complement titration, that was unexplainable from the standpoint of the pH or the reagents used.

Amboceptor titrations resulted in a unit well within the usable range, but the complement titration was unsatisfactory for use, with complete hemolysis in perhaps only the first one or two tubes.

All guinea pigs used for complement had been tested previously for titer and complement fixation and found to be satisfactory. Blood from five guinea pigs was pooled. Since lyophilized complement titrations occasionally exhibited similar reactions, complement per se was eliminated as a possible cause of the difficulty. Inhibition of hemolysis was noted in the complement titrations containing antigen. The pH of the saline was always within usable range.

The absence of albumin in the titration was considered as a possible cause of the difficulty encountered. Varying amounts of pooled negative serum were found to be unsatisfactory as a source of albumin, but the addition to each tube in the titration of 0.2 c.c. of 50 per cent egg albumin in 0.85 per cent saline, as previously recommended by Kolmer for testing spinal fluids, was found satisfactory.

Parallel titrations using the same reagents, except for the absence of the 0.2 c.c. of 50 per cent egg albumin in 0.85 per cent saline, resulted in the titrations given in Table I.

Supplementary titrations following the eighteen hour incubation of the complement fixation tests rarely result in a change of amboceptor dilution when the units for the test have been determined by a preliminary titration using the 0.2 c.c. amounts of 50 per cent egg albumin in each tube of the titration; whereas, the supplementary titrations after the incubation period, on tests in which the unit of complement has been determined in preliminary titrations not using the egg albumin, frequently necessitate an increased dilution of amboceptor for tests with a maximum sensitivity.

Variations in the unit of complement with cardiolipin-lecithin antigen and regular antigen have been reported previously[1] and the variation is consistent whether or not egg albumin is used.

*Pathologist, Santa Rosa Hospital Laboratories.
†Serologist, Santa Rosa Hospital Laboratories.
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TABLE I

WITH EGG		WITHOUT EGG	
AMBOCEPTOR 1 UNIT	COMPLEMENT	AMBOCEPTOR 1 UNIT	COMPLEMENT
10,000	1:37	10,000	1:30
12,000	1:43	12,000	1:30
12,000	1:33	12,000	Only .5 tube hemolyzed
12,000	1:43	12,000	1:30
12,000	1:37	12,000	1:33
12,000	1:43	12,000	1:33
12,000	1:43 Kolmer 1:50 Cardio-Kolmer	12,000	1:37
16,000	1:33	16,000	1:30
16,000	1:43	16,000	1:30
16,000	1:43	10,000	1:33
16,000	1:33	12,000	1:33
16,000	1:43 Kolmer 1:50 Cardio-Kolmer	12,000	1:33
16,000	1:37	16,000	1:33
16,000	1:43	16,000	1:33
16,000	1:43 Kolmer 1:50 Cardio-Kolmer	16,000	1:33
16,000	1:50	16,000	1:33
16,000	1:37	16,000	1:37
16,000	1:43	16,000	1:37
16,000	1:50	16,000	1:37
20,000	1:37	20,000	1:30
20,000	1:43	20,000	1:30
20,000	1:33 Kolmer 1:37 Cardio-Kolmer	20,000	1:33 Kolmer 1:33 Cardio-Kolmer
20,000	1:43 Kolmer 1:50 Cardio-Kolmer	10,000	1:37
20,000	1:37 Kolmer 1:43 Cardio-Kolmer	20,000	1:37

CONCLUSION

The use of 0.2 c.c. of 50 per cent egg albumin in normal saline in each tube of the preliminary amboceptor and complement Kolmer titrations usually results in a higher unit of complement and frequently in an increased dilution of amboceptor to be used in the Kolmer complement fixation test for syphilis. With the addition of the 50 per cent egg albumin, supplementary titrations following the incubation period rarely result in the necessity for a change in the amboceptor dilution to obtain maximum sensitivity.

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A COLORIMETRIC METHOD FOR THE DETERMINATION OF MICROQUANTITIES OF ETHANOL IN BLOOD AND OTHER BIOLOGIC FLUIDS

RICHARD J. HENRY, M.D., CAROL F. KIRKWOOD, A.B., SAM BERKMAN, PH.D., RILEY D. HOUSEWRIGHT, PH.D., AND JANE HENRY, PH.D.
FREDERICK, MD.
WITH THE TECHNICAL ASSISTANCE OF RENATE HIRSCH

THE principal methods for the determination of microquantities of ethanol involve its oxidation followed by measurement of the resultant equivalent reduction. Various oxidizing agents have been used[1-3] but the majority of methods are based on the work of Widmark[4] and Nicloux and co-workers[5] and use an acid solution of potassium dichromate. Investigators have varied the procedure of distilling the alcohol into the acid dichromate, and both titrimetric[6-8] and colorimetric[9-11] methods have been used to determine the reduced dichromate. The maximal sensitivity of these methods is approximately 20 μg of ethanol. These methods are not specific, since any volatile substance oxidizable by dichromate will be determined as ethanol. The presence of such substances as methanol, formaldehyde, acetone, and other aldehydes and ketones gives high results.[1, 6, 12, 13] The accuracy of these methods in the determination of ethanol is open to question unless such substances can be removed or are known not to be present in quantities causing interference. This is especially important in the determination of the ethanol level in blood for medicolegal purposes.

A new method for the determination of ethanol has been devised involving the conversion of ethanol to acetaldehyde and the determination of the latter colorimetrically with p-hydroxydiphenyl.[14, 15] This method is considerably more sensitive and more specific than pre-existing methods. Furthermore it retains the advantages of requiring no special apparatus and of being simple to carry out.

PROCEDURE

Up to 20 c.c. of the sample to be analyzed are placed with several glass beads in a 50 c.c. single side arm distillation flask connected with a small Liebig condenser. Distilled water is added to 20 c.c., followed by 0.1 c.c. 10 per cent NaOH. The flask is closed tightly with a rubber stopper and at least 5 c.c. distilled. The entire distillate is then washed into the flask of an identical setup, the flask in this case containing 2.5 c.c. saturated aqueous $K_2Cr_2O_7$ (C.P.), 0.5 c.c. concentrated H_2SO_4 (C.P.), and several glass beads. (Care must be taken to prevent any of the dichromate from entering the side arm since dichromate interferes with the color reaction.) The flask is stoppered immediately with a rubber stopper and the acetaldehyde formed is distilled into a graduated receptacle, such as a centrifuge tube, in an ice bath.* The volume that must be distilled to obtain all the

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*What percentage of the ethanol was oxidized to acetic acid was not determined. Consistency of the results obtained indicates that the fraction of the ethanol completely oxidized remained constant under the conditions of the procedure outlined. Distillation from the dichromate-sulfuric acid mixture was begun immediately after addition of the ethanol, and no differences in results were observed whether this mixture was hot or cold at the time of addition of the ethanol. As soon as one sample has been distilled, another can be added to the reaction mixture. Thus several samples can be distilled in succession without changing the dichromate-sulfuric acid mixture.

acetaldehyde formed varies directly with the amount of ethanol present in the original sample. It was found that if the original sample contains 20 μg of ethanol at least 5 c.c. must be distilled; for 10 μg of ethanol a distillation of 2 c.c. suffices.

The acetaldehyde in the distillate is then determined colorimetrically by reaction with p-hydroxydiphenyl.[14, 15] One cubic centimeter of the distillate is placed in an 18 by 150 mm. Pyrex test tube. One drop of 5 per cent $CuSo_4$ is added, followed by 6.00 c.c. of concentrated H_2SO_4 (special, As- and N-free) added slowly, with shaking, in an ice bath. One-tenth cubic centimeter of 1.5 per cent p-hydroxydiphenyl in 0.5 per cent NaOH is added directly to the solution and dispersed by vigorous shaking. The tube is placed in a 30° C. water bath and after approximately fifteen minutes the reagent is redispersed by shaking. After incubation for at least thirty minutes the tube is placed in a boiling water bath for ninety seconds to dissolve excess reagent. The tube is then placed immediately in an ice bath and brought to room temperature. The color (deep violet) is read in a photoelectric colorimeter against a sulfuric acid-reagent blank which has been treated as described (except that 1.00 c.c. of water is used instead of the distillate). A filter having peak transmittance at approximately 560 mμ is used. The color can be read immediately and is stable for several hours.

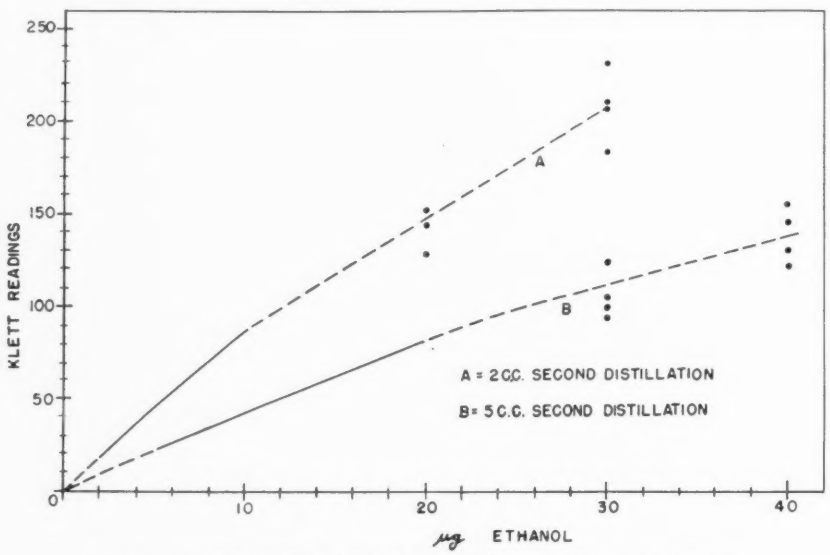

Fig. 1.—Color intensity obtained with known quantities of ethanol.

In our experiments the Klett-Summerson photoelectric colorimeter was used with a Klett No. 54 filter. Fig. 1 presents graphically the results of distilling 2 and 5 c.c. quantities in the second distillations, using known concentrations of ethanol. The solid portions of the curves were drawn from the experimental points (20 for curve *A*, 32 for curve *B*) by the method of least squares. It is apparent from the graph that the maximal amounts of ethanol falling on the straight line (solid) portions of the curves for the 2 and 5 c.c. distillations are approximately 10 and 20 μg, respectively. Several experimental points above these limits are plotted in Fig. 1 to show the variability of results obtained at higher concentrations.

Sensitivity and Precision.—The sensitivities for the 5 and 2 c.c. distillations are approximately 1 and 0.5 μg of ethanol, respectively. It was found that the sensitivity could be increased to approximately 0.2 μg by distilling only 1.20 c.c. in the second distillation. Thus, if a 10 c.c. sample is available a concen-

tration of 0.02 μg per cubic centimeter can be determined. The standard error of a single determination in the range between 5 and 20 μg of ethanol for the 5 c.c. distillation is 5 per cent and between 2 and 10 μg of ethanol for the 2 c.c. distillation, 7 per cent. Since nineteen times out of twenty a single determination will be within ± 2 standard errors of the true value, the error of a single determination is ± 10 per cent for the 5 c.c. distillation and ± 14 per cent for the 2 c.c. distillation.* (The experimental error is greater at or near the limit of sensitivity.)

The error, of course, can be reduced by running replicates. The number of replicates required (N) to give a desired precision (P) can be computed by substitution in the following formulas.

$$N = \frac{100}{(P)^2} \quad \text{(for 5 c.c. second distillation)}$$

$$N = \frac{196}{(P)^2} \quad \text{(for 2 c.c. second distillation)}$$

Interfering Substances.—The following substances did not give color in 100 μg amounts, distilling 2 c.c. in the second distillation: dihydroxyacetone, fructose, d-xylose, d-ribose, succinic acid, fumaric acid, citric acid, malic acid, cis-aconitic acid, α-ketoglutaric acid, acetone, ascorbic acid, tartaric acid, urea, glycine, trytophane, methionine, methanol, glucose, pyruvic acid, glycerol, acetic acid, and lactic acid (up to 500 μg).

In Table I are listed those substances which were found to give color, distilling 2 c.c. on the second distillation. One hundred microgram amounts were used and the interference is given in terms of micrograms of ethanol giving an equivalent intensity of color. Obviously, acetaldehyde itself would give the color reaction.

TABLE I. SUBSTANCES INTERFERING IN THE DETERMINATION OF ETHANOL

INTERFERING SUBSTANCE	COLOR YIELDED BY 100 μG IN TERMS OF μG ETHANOL
Oxalacetic acid	3
α-Glycerophosphate	20
Glyceraldehyde	1
n-Propyl alcohol	22 (blue color)
n-Butyl alcohol	20 (purple color)
Isobutyl alcohol	2
Allyl alcohol	10 (red color)
n-Amyl alcohol	6
Isoamyl alcohol	6

In many instances the first distillation procedure could be omitted. It was found, however, that lactic acid interfered and that this interference was quite variable. After several attempts, the only procedure found effective in eliminating lactic acid interference was the preliminary distillation from basic solution.

*A good portion of this error undoubtedly is dependent on the accuracy with which the second distillate is measured (the second distillates were collected and the volume measured in graduated centrifuge tubes) and on the variation in the Klett colorimeter tubes used (standardized to ± 3 Klett scale readings).

Determination of Ethanol in Blood.—The blood level of ethanol important from the medicolegal aspect centers around 2 mg. per cubic centimeter. Ethanol was added to a concentration of 1.00 mg. per cubic centimeter to samples of whole citrated blood from ten individuals. One-tenth cubic centimeter of the blood-alcohol mixture was diluted to 5.00 cubic centimeters. Four and one-half cubic centimeters of N/12 H_2SO_4 and 0.50 c.c. of 10 per cent $Na_2WO_4 \cdot 2H_2O$ were added, making a total volume of 10.00 cubic centimeters. The ethanol in 1.00 c.c. of the supernatant was determined by the procedure described. To eliminate error arising from variation in the colorimeter tubes, a single tube was used in the blood determinations for all colorimeter readings. Blood samples analyzed after standing overnight gave the same results as those analyzed immediately after the addition of ethanol. The average recovery for forty-two determinations on these samples was 98.6 per cent. The standard error of a single determination was ± 3 per cent, indicating that nineteen times out of twenty the value obtained from a single determination will be within ± 6 per cent of the true value.

Without preliminary precipitation of blood proteins and erythrocytes with tungstic acid, very high blank readings were obtained, presumably due partially or totally to acetaldehyde in the erythrocytes.[16] Similarly, high blanks were obtained if the first distillation was omitted.

Values for the normal ethanol content of blood reported in the literature range from 4 to 40 μg per cubic centimeter[1, 3, 17] and those for interfering substances estimated as ethanol have been reported as 40 μg per cubic centimeter.[18] In our experiments blank determinations (without added ethanol) on blood samples from thirteen normal individuals gave values from 1 to 10 μg per cubic centimeter, with an average of 6 μg per cubic centimeter. There is no assurance that these values represent the true normal ethanol level since this method of determination is not absolutely specific. Such levels obviously do not interfere in the determination of ethanol concentrations in blood important from a medicolegal aspect.

The extreme sensitivity of this method makes it possible to do blood alcohols on the amount of blood obtainable from finger puncture. The collection and initial dilution of the blood could be carried out with a white blood cell dilution pipette (giving a dilution of 1:20). Before this could be accepted from a medicolegal aspect it would be necessary to ascertain whether the blood alcohol level is the same in blood obtained by finger puncture as in the blood obtained by venipuncture.

It is noteworthy that methanol and acetone do not interfere in this test.

SUMMARY

A method for the determination of ethanol in blood and other biologic fluids is described based on oxidation of the alcohol to acetaldehyde with subsequent determination of the latter photocolorimetrically with p-hydroxydiphenyl reagent. This method is more sensitive than pre-existing methods and has a precision of approximately ± 6 per cent on a single blood determination.

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THE MICROCOLORIMETRIC DETERMINATION OF SODIUM IN HUMAN BIOLOGIC FLUIDS

ANTHONY A. ALBANESE, PH.D., AND MARILYN LEIN
NEW YORK, N. Y.

BARBER and Kolthoff[1] in 1928 described the use of the triple salt, uranyl zinc sodium acetate, for the gravimetric determination of sodium. Subsequently Butler and Tuthill[2] applied this method with modifications to the determination of sodium in biologic products. Although the gravimetric procedure gives good results, its operation is tedious and requires large amounts of often scarce biologic material. More recently one or both of these shortcomings have been overcome in part by treatment of the precipitate by various colorimetric[3] or titrimetric[4] reactions. In using these more rapid but also more complex techniques, we gained the impression that greater ease of operation would be achieved if the microquantities of sodium contained in 0.2 c.c. of serum or plasma could be measured in terms of the yellow color of the triple salt complex. Examination of the literature showed that in 1929 Caley and Foulk[5] had applied this principle to inorganic samples with limited success. Apparently the large amounts of material requisite for the procedure and the difficulties embodied in measuring the density of the resultant yellow color by the then used optical colorimeters discouraged the further application of this ideally simple procedure to inorganic materials[6] and biologic specimens. The excellent results which we obtained in preliminary tests of this principle in which the Klett-Summerson photoelectric colorimeter was employed for measuring the density of the yellow color of solutions containing varying amounts of the sodium zinc uranyl acetate complex prompted us to attempt the adaptation of this technique to the determination of sodium in biologic fluids. The analytic adequacy of the method which was evolved as the result of this effort was amply shown by recovery and reproducibility tests. The concordant results obtained with this rapid technique when employing 0.2 c.c. samples of urine, cerebrospinal fluid, and various blood fractions recommends its use for pediatric studies.

EXPERIMENTAL

Reagents.—

Uranyl zinc acetate:[1] 10 Gm. of uranyl acetate were dissolved in 50 c.c. of boiling water containing 2 c.c. of glacial acetic acid. In another flask 30 Gm. of zinc acetate were dissolved in 50 c.c. of boiling water containing 1 c.c. of glacial acetic acid. The boiling solutions were mixed and again heated just to boiling. After standing overnight at room temperature, the resultant solution was filtered by gravity and mixed with an equal volume of 95 per cent ethanol.

From the Department of Pediatrics, New York University College of Medicine and the Children's Medical Service, Bellevue Hospital.
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This solution was refrigerated at 4° C. for forty-eight hours and then filtered by gravity for use. The reagent is stable at room temperature.

Ethanol: 95 per cent by volume.

Trichloracetic acid: 20 per cent solution.

Sodium standard: 508.4 mg. of oven dried (100° C.) sodium chloride were dissolved in 100 c.c. of triple distilled water; 1 c.c. of this solution is equivalent to 2 mg. of sodium.

DETAILS OF PROCEDURE

In order to determine the colorimeter filter best suited for the photoelectric measurement of the yellow color of sodium uranyl zinc acetate, the spectral transmission of solutions of the salt equivalent to 1 mg. of sodium was measured in a Coleman Universal spectrophotometer, Model 11. These data indicated that

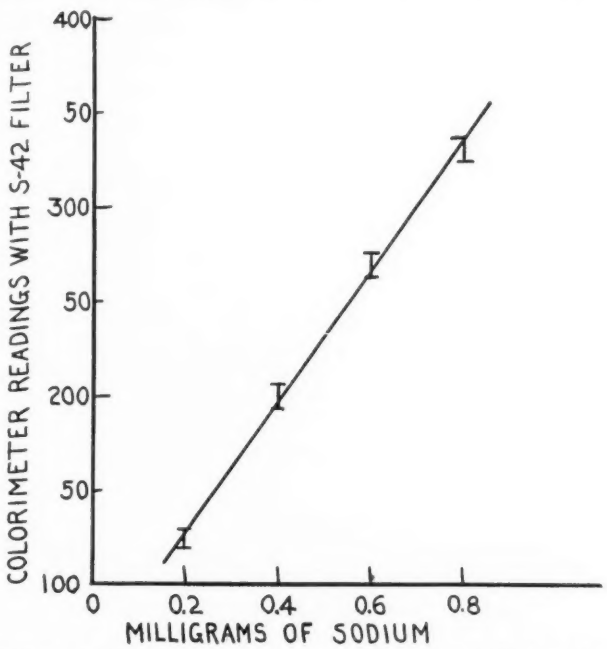

Fig. 1.—Relation of color intensity to amount of sodium. Each point represents the average of five determinations. The bars above and below each point indicate the deviation range of the readings.

maximum optical density for this colored solution occurs in the region 420 to 440 μ and that to realize maximum sensitivity for the test the readings should be made with filter S-42.

The analytical efficacy of the scheme was ascertained as follows. Suitable aliquots (0.1 to 0.4 c.c.) of the standard in 15 c.c. graduated centrifuge tubes were each treated with 1 c.c. of the reagent. After refrigeration for one hour at 4° C. the samples were centrifuged and the supernatant fluid decanted and discarded. The yellow precipitate was washed by centrifugation with 2 c.c. of 95 per cent ethanol and dissolved in 5 c.c. of water. These solutions were transferred to the colorimeter tubes and their density measured in the Klett-

Summerson photoelectric colorimeter using filter S-42. The results of this experiment, which are shown in Fig. 1, demonstrate that the color intensity of the solution varies in accordance with Beer's law for this range of sodium concentration. This color was found to be stable for several hours.

The deviations of the color readings from the mean indicate that the experimental error of the method is approximately ±5 per cent, which is no greater, if not less, than that incurred in the gravimetric, titrimetric, or other colorimetric methods.

The adequacy of the one hour of refrigeration and the single alcohol washing employed in these tests was demonstrated by the concordance of the readings of samples of the standard so obtained with those of similar samples which were allowed to stand in the refrigerator overnight and which were washed three times with 95 per cent ethanol.

Since the application of the method to urine was expected to entail the removal of phosphate ions, solutions of KH_2PO_4 which contained the interfering ions in more than twice their normal concentration in the urine were treated in the manner described for the sodium standard. This test disclosed that the precipitate obtained under these conditions, unlike the sodium acetate complex, was insoluble in water, could be removed by centrifugation, and did not contribute any measurable color to the supernatant fluid. This observation indicated that the urinary phosphates could be readily disposed of in this manner rather than by the use of supplementary reagents.[2] The analytical adequacy of removing phosphate ions by this scheme was further shown by the agreement of sodium values of 0.2 and 0.3 c.c. aliquots of the standard before and after the addition of 2 mg. of KH_2PO_4 (Table I). This amount of phosphate ion is equivalent to approximately 5 Gm. of this ion per liter or about twice its normal concentration in human urine.

APPLICATION OF METHOD TO BIOLOGIC MATERIALS

In applying the technique to urine derived from adults and children the following procedure was found satisfactory: to 0.2 c.c. of urine in a 15 c.c. centrifuge tube was added, with mixing, 1 c.c. of reagent. After refrigeration for one hour at 4° C. the mixture was centrifuged at 3,000 revolutions per minute for ten minutes. The supernatant fluid was discarded and the tubes were carefully drained by inversion. The yellow precipitate was resuspended in 2 c.c. of 95 per cent alcohol, washed by centrifugation, and again drained by inversion. The precipitate was dissolved in 5 c.c. of distilled water. (The turbidity which may result from the presence of an excess of phosphate ions is easily removed by centrifugation.) The clear yellow supernatant fluid was decanted into the colorimeter tubes and the intensity of the yellow color measured in the Klett-Summerson colorimeter using an S-42 filter. A parallel determination was also done on a 0.2 c.c. aliquot of the standard. The sodium content per cubic centimeter of urine was obtained from the following formula:

$$\text{Reading of unknown/reading of standard} \times 0.4 \text{ mg. of Na} \times 5$$

It is obvious that careless handling of the tubes during the washing processes will result in the loss of precipitate which will lead to serious analytic errors. For this reason all determinations should be done in duplicate.

The sodium content of spinal fluid also can be determined directly in 0.2 c.c. samples as described for urine. The presence of the small amounts of phosphate ions and protein which usually occur in this material does not interfere with this assay.

TABLE I. RECOVERY OF ADDED SODIUM

SAMPLE	SODIUM ADDED (MG.)	SODIUM FOUND (MG.)	RECOVERY OF ADDED SODIUM (%)
KH_2PO_4, 2 mg.	0	0	
KH_2PO_4, 2 mg.	0.4	0.4	100
KH_2PO_4, 2 mg.	0.6	0.6	100
Urine P, 0.2 c.c.	0	0.129	
Urine P, 0.2 c.c.	0.2	0.327	99
Urine G, 0.2 c.c.	0	0.557	
Urine G, 0.2 c.c.	0.2	0.748	95.5
Urine M, 0.2 c.c.	0	0.473	
Urine M, 0.2 c.c.	0.2	0.668	97.5
Serum D, 0.1 c.c.	0	0.315	
Serum D, 0.1 c.c.	0.2	0.520	102
Serum W, 0.1 c.c.	0	0.308	
Serum W, 0.1 c.c.	0.2	0.515	103
Plasma C, 0.1 c.c.	0	0.362	
Plasma C, 0.1 c.c.	0.2	0.562	100

In the analysis of serums, plasmas, or whole blood, 0.2 c.c. aliquots are treated with 0.6 c.c. of 20 per cent trichloracetic acid and, after centrifugation, 0.4 c.c. of the supernatant fluid (equivalent to 0.1 c.c. of the original sample) is treated with 1 c.c. of the reagent and the determination completed as described for urine. In practice, whenever sufficient material is available, it is more convenient for duplicate analyses to obtain two 0.4 c.c. aliquots from 0.5

TABLE II. SODIUM CONTENT OF VARIOUS HUMAN BIOLOGIC FLUIDS

SUBJECT	SAMPLE	SODIUM CONTENT
C, Adult, male	Serum	316 mg. %
E, Adult, female	Plasma	307 mg. %
H, Adult, male	Whole blood	224 mg. %
J, Infant, female	Serum	325 mg. %
W, Infant, male	Cerebrospinal fluid	285 mg. %
D, Infant, female	Cerebrospinal fluid	312 mg. %
N, Infant, female	Cerebrospinal fluid	294 mg. %
J, Premature infant, male	Urine	.129 Gm./day
G, Infant, male	Urine	.314 Gm./day
S, Infant, male	Urine	.550 Gm./day
A, Adult, male	Urine	3.75 Gm./day
M, Adult, female	Urine	2.37 Gm./day

c.c. portions of the blood fractions to which are added 1.5 c.c. of the trichloracetic acid solution. The adequacy of the technique as applied to these biologic fluids is indicated by the quantitative recovery of added sodium (Table I).

The results of analyses of biologic fluids obtained from human subjects of various ages are given in Table II. It will be noted that these are in the range of the values reported by others.

SUMMARY

A rapid microcolorimetric procedure for the estimation of the sodium content of biologic fluids has been described which yields values comparable to those of methods requiring larger amounts of material and greater expenditure of time.

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A NEW COLORIMETRIC METHOD FOR THE DETERMINATION OF PREGNANDIOL

JOSEPH W. GOLDZIEHER, M.D.*
NEW YORK, N. Y.

THE earliest methods for the quantitative determination of pregnandiol were gravimetric. Some years later Talbot and co-workers[1] described a colorimetric technique: the addition of sulfuric acid to pregnandiol produced a color with a maximum absorption at 420 millimicrons. The accuracy of the method was reported to be within 3 per cent for quantities of pregnandiol between 0.05 and 0.3 milligrams. The relationship between color density and quantity of pregnandiol was according to the equation $C = 2 - \frac{\log G}{K}$. Although this reaction was entirely satisfactory in the hands of Talbot and co-workers,[1] others[2, 3] found that it shared some of the well-known, unpleasant habits of sulfuric acid colors in general. The Liebermann-Burchardt reaction, for instance, has a tendency to develop adventitious tints which may be most disturbing; other colorimetric methods using sulfuric acid often have been replaced for the same reason. Guterman[3] found spurious colors so troublesome during his pregnandiol determinations that it became necessary for him to modify his technique considerably and to prepare fresh reagents every two to three days.

In view of these facts an attempt was made to develop another color reaction which would be at least as sensitive as the sulfuric acid method, yet which would not develop adventitious colors. Of a number of reactions tested, one in particular gave indications of being suitable. It has been reported[4] that cholesterol produces a characteristic color when added to acetyl chloride and zinc chloride in a solution of glacial acetic acid. Subsequent workers[5-7] developed this reaction into a quantitative method for the determination of blood cholesterol; in our experience it has distinct advantages over the usual Bloor method. Despite certain theoretic predictions it was found that pregnandiol gives a stable, quantitative color reaction with acetyl chloride and zinc chloride; within certain limits the quantity of pregnandiol and the color intensity have a straight-line relationship, so that direct colorimetric readings can be made.

EXPERIMENTAL

Carefully recrystallized pregnandiol was used throughout. Ether-alcohol solutions containing 0.10 and 1.0 mg. per milliliter were prepared; the desired quantity was pipetted into the reaction vessel and the solvent evaporated. The

From the Endocrine Division of the Department of Obstetrics and Gynecology, Duke University School of Medicine and Duke Hospital, Durham, N. C.

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*St. Clare's Hospital.

zinc chloride reagent was prepared by dissolving 38 Gm. of pure zinc chloride in sufficient glacial acetic acid to make a final volume of 100 milliliters.

The absorption spectrum of the color complex, after development for thirty minutes at 50° C., is shown in Fig. 1. Maximum absorption occurred between 370 and 450 millimicrons. At wave lengths below 300 mμ the solvent became too opaque for further determinations.

Various factors which might affect the color development, such as proportion of reagents, temperature, time, and concentration, were studied.

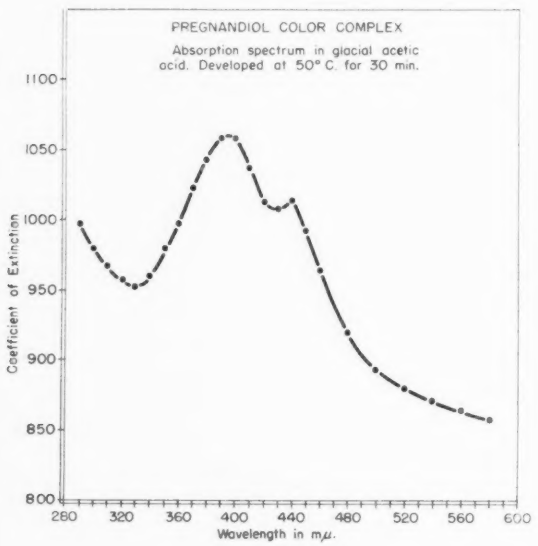

Fig. 1.

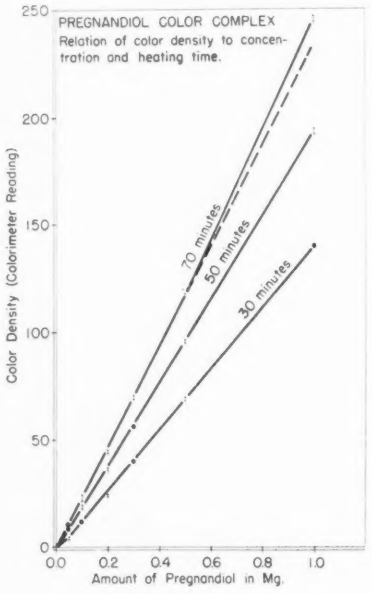

Fig. 2.

It was found that 2.5 c.c. of acetyl chloride were most suitable to a final volume of 10 c.c. attained by the addition of zinc chloride reagent.

The highest temperature at which color development could be carried out without substantial losses of acetyl chloride by volatilization was found to be 50° C.

Color development at a constant temperature of 50 ± 2° C. proceeds rapidly during the first fifty minutes. Small quantities of pregnandiol (less than 0.2 mg.) attain a constant color density by 150 minutes, but with larger amounts (0.3 to 1.0 mg.) the color continues to increase in density even after 300 minutes of incubation. When cooled to room temperature the color is stable and remains constant for at least two and one-half hours.

A different aspect of the relationship between color density, concentration and incubation time is shown in Fig. 2. The data are taken from runs carried out in triplicate. It will be seen that color versus concentration is a straight-line relationship if the heating time is thirty to fifty minutes; incubation for longer than sixty minutes yields aberrant results with higher concentrations of pregnandiol.

It was found impracticable to control the temperature and the heating so closely that the use of a standard solution became unnecessary. With the use of a standard (0.5 mg. pregnandiol) the method was found to be accurate within 4 per cent.

PROCEDURE

The unknown quantity of pregnandiol is dissolved in a suitable amount of solvent, such as ether-alcohol, and an aliquot representing approximately 0.1 to 1.0 mg. is pipetted into a 10 c.c. glass-stoppered volumetric flask. The solvent is evaporated off. Approximately 6 c.c. of zinc chloride reagent are placed in the flask and 2.5 c.c. of acetyl chloride are added from a small burette. A standard for comparison, representing 0.50 mg. of pregnandiol, is prepared in similar fashion. The flasks are incubated in a hot water bath at 50° C. for thirty minutes. The flasks are then dipped briefly in ice water until cool and are allowed to stand at room temperature for twenty minutes. The volume is adjusted to 10 c.c. with zinc chloride reagent. After careful mixing of the solution to insure color uniformity, it is ready for reading.

SUMMARY

A new colorimetric reaction with pregnandiol, based on its interaction with acetyl chloride and zinc chloride in glacial acetic acid solution, is described. The characteristics of the reaction and the technical procedure are discussed briefly.

We express our thanks to Dr. W. J. Dann of the Department of Biochemistry for the spectrophotometric determinations.

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THE EFFECT OF CHLORINE ON COMPLEMENT FIXATION

ANNE C. KIMBALL, PH.D., AND PAUL W. KABLER, PH.D., M.D.
MINNEAPOLIS, MINN.

MANY laboratories are equipped with continuous-flow water stills which permit considerable quantities of chlorine in the tap water to be condensed in the distillate. In our experience with a Barnstad "Q" type of still, the chlorine content of the distillate may be as high as the water from which it is prepared, and may, therefore, fluctuate in relation to the chlorine residual of the undistilled water.

When this still was first put into use the distillate was used in preparing saline for the Kolmer-Wassermann test and we were not able to get a complement titration in the approved dilution range even though all the biologic reagents had been satisfactory when titrated with saline of distillates from other stills. This quality of interfering with the complement titrations was found to correlate with the chlorine residual of the various distillates.

Directions for making the saline for Kolmer's modification of the Wassermann test specify the use of 0.85 per cent sodium chloride. The addition of 1.0 c.c. of 10 per cent magnesium sulfate to each liter is recommended but is not always essential. Heating in an Arnold sterilizer for one hour is considered advisable if the saline is not used immediately.[1, 2] No specific criteria of acceptable water are given in reference to chlorine residual.

High Chlorine Residual (Above 0.8 Parts Per Million).—When the chlorine residual of the water is 0.8 parts per million or higher, complete hemolysis is not obtained in any tubes of the hemolytic titration using Kolmer's method. Sheep cell suspensions using saline with these high chlorine residuals show a slight change in color, appearing darker and somewhat brown. At times even lower chlorine residuals will prevent complete hemolysis in all of the titration tubes.

Study of Lower Chlorine Residuals (0.6 Parts Per Million or Less).—The total chlorine residual of freshly distilled water was determined with a comparator using the orthotoludine method.[3] Chlorine-free water (Table I, A) was obtained by autoclaving a portion of the water for twenty minutes at 15 pounds, once or twice as needed. Water containing three intermediate amounts of chlorine (B, C, and D) was prepared by mixing the chlorine-free (A) and the chlorine-containing (E) water.

Five 0.85 per cent saline solutions with magnesium sulfate added, as recommended by Kolmer, were prepared using distilled water A, B, C, D, and E (as in the preceding paragraph). All reagents for use in the Kolmer-Wassermann test were prepared with the five saline solutions and five titrations and quantitative tests were set up. In the first set of experiments five portions of

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sheep cells were washed and packed separately with the five saline solutions of varying chlorine residuals. For the second set of experiments the sheep cells were washed, packed, and diluted to 20 per cent with chlorine-free saline, and five portions were further diluted to 2 per cent with the varying salines. The same Kolmer antigen and hemolysin were used in both experiments, while the Lyovac complement and positive sera were different.

The results of the ten titrations and quantitative tests are shown in Table I. It is to be noted that with each increase in chlorine residual, more hemolysin or more complement or more of both is required for the tests as indicated by the titration.

TABLE I

EXPERIMENT	SOLUTION	CHLORINE RESIDUAL (P.P.M.)	HEMOLYSIN TITRATION	ACTIVE UNIT OF COMPLEMENT IN ML. OF 1:30 DILUTION	QUANTITATIVE KOLMER-WASSERMANN* (ML. OF POSITIVE SERUM)					
					0.2	0.1	0.05	0.025	0.0125	CONTROL 0.2
I										
	A	0	1-5000	0.4	4	4	4	4(3)	±(−)	−
	B	.15	1-5000	0.45	4	4	4	4(3)	±(−)	−
	C	.3	1-5000	0.5	4	4	4	4(2)	1(−)	−
	D	.45	1-4000	0.5	4	4	4	4(2)	±(−)	−
	E	.6	1-3000	0.5	4	4	4	4(1)	±(−)	−
II										
	A	0	1-8000	0.35	4	4	3(1)	−	−	−
	B	.12	1-6000	0.35	4	4	3(1)	−	−	−
	C	.25	1-5000	0.4	4	4	3(1)	−	−	−
	D	.37	1-4000	0.5	4	3(1)	1(−)	−	−	−
	E	.5	1-4000	0.5	4	3(1)	−	−	−	−

*Readings made ten minutes after clearing of controls.

Reading made after one hour of incubation is shown in parenthesis if different from the earlier reading.

Quantitative tests were set up using four different positive sera on each day. The results with only one sera are shown in Table I. The results were similar with all sera, that is slightly weaker reactions with the chlorine-containing saline.

DISCUSSION

These results indicate that saline made with chlorine-free distilled water should be used for diluting reagents for complement fixation tests. The advantages are as follows:

1. Removal of one factor which may make it impossible to get a titration in the approved range.

2. Higher sensitivity of tests.

3. Lower cost of reagents; this is particularly important in reference to the complement.

It is interesting to note that the actual tests do not indicate that the chlorine has an enhanced deleterious effect on the complement during the long icebox fixation. The explanation of this is not apparent from the present observations. This study included only the Kolmer-Wassermann complement

fixation test, but it seems reasonable to assume that the same effect would be seen with any complement fixation procedure.

Distilled water containing chlorine may be rendered chlorine-free by several methods.

1. Storage for one to several months
2. Boiling to two-thirds or one-half the original volume
3. Autoclaving at fifteen pounds pressure for twenty minutes once or twice as needed
4. Inserting an activated-carbon filter to remove the chlorine from water going to the still

Before use, the water must be tested for chlorine residual. Since a satisfactory water must be chlorine-free, there is no need to determine the content if present.

CONCLUSIONS

1. Attempts to titrate the reagents for the complement fixation test are unsuccessful when distilled water with a high chlorine residual (0.8 parts per million or higher) is used in preparing the saline solution.

2. Complement fixation tests using water with lower chlorine residuals (0.6 parts per million or less) require more hemolysin or more complement or more of both than tests using chlorine-free water.

3. Only chlorine-free water should be used in saline for complement fixation procedures.

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